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Interactions of intestinal epithelial cells with bacterial extracellular products

Daniel Adam Patten BSc. (Hons), MSc.



University of
HUDDERSFIELD

**A thesis submitted to the University of Huddersfield
in partial fulfilment of the requirements for
the degree of Doctor of Philosophy**

The University of Huddersfield

June 2013

Abstract

University of Huddersfield

ABSTRACT OF THESIS submitted by Mr Daniel Adam Patten for the Degree of PhD

The enteric microflora represents one of the densest microbial populations in the biological world; as a consequence, the intestinal immune system is constantly exposed to high concentrations of antigenic materials. One of the major frontline defences in the innate immune system is the intestinal epithelial layer, which presents both a physical barrier and an immune sensor to the antigens of the lumen. The latter function is performed by the expression of pattern recognition receptors, which recognise a wide variety of bacterial antigens, and the production of inflammatory cytokines, which stimulate, or inhibit, inflammation. The overall aim of the present study was to investigate the immunomodulatory potential of extracellular products, from non-pathogenic bacteria, with intestinal epithelial cells.

Two *in vitro* human intestinal epithelial cell lines HT29-19A and Caco-2 were shown to exhibit different expression levels of Toll-like receptors (TLRs) and the inflammatory cytokines, interleukin (IL)-8 and IL-10. These differences were reflected in their sensitivity (monitored by IL-8 release) to known TLR agonists, isolated from pathogenic bacteria. Caco-2 cells were also shown to form physiologically active tight junctions, with the formation and maintenance of domes. Both cell lines exhibited sensitivity to the cytotoxic extracellular products of the enteropathogen *Clostridium difficile*. Extracellular products, in crude cell-free supernatants and bacterial sonicates, from the commensal Gram-negative bacterium *Escherichia coli* C25, significantly increased IL-8 release in both cell lines. Lipopolysaccharides and membrane vesicles were shown to contribute to the proinflammatory effects of C25-derived extracellular products. These extracellular products were also shown to regulate bacterial internalisation in both cell lines. Crude cell-free supernatants and bacterial sonicates from two lactobacilli strains *Lactobacillus acidophilus* 5e2 and *Lactobacillus helveticus* sp. Rosyjski were also found to be biologically active, stimulating IL-8 release and TLR expression modification in both intestinal epithelial cell lines. In addition, ultrapure EPSs, isolated from these lactobacilli strains, were also found to possess immunomodulatory potential. HT29-19A cells, pre-treated with EPSs, were found to be 'primed' to bacterial agonists, peptidoglycan and flagellin, with a significantly potentiated release in IL-8 observed. Finally, EPSs were also found to modify bacterial adherence and internalisation in both cell lines.

In conclusion, data presented in this investigation has shown that the use of the intestinal epithelial cell lines, HT29-19A and Caco-2, presents a reasonable model for investigating the interaction of bacterial extracellular products with the intestinal epithelium. Additionally, it has demonstrated that extracellular products, isolated from non-pathogenic, enteric-associated bacteria, possess immunomodulatory potential *in vitro*. If these effects were also to occur *in vivo*, then they could potentially contribute to intestinal homeostasis and the innate 'priming' of the epithelial layer to pathogens and their products.

Acknowledgements

First and foremost, I would like to thank my supervisor, mentor and now friend, Dr. Andrew Collett, for his help, guidance and advice in both work and general life matters; he has helped me develop a great deal, both as a researcher, and as a person. Also, a big ‘thank you’ to Dr. Paul Humphreys who has not only supplied bacterial strains and other lab materials used in this project, but has been has also been an enduring source of knowledge and advice in all matters bacterial. A ‘thank you’ must also go to the Biology Technicians who helped me more times than I care to mention.

I would also like to thank all my friends and colleagues in Huddersfield, who have supported and consoled me (both with copious amounts of alcohol!) throughout the ups and downs of PhD life...it has definitely been emotional (probably booze-related!)! Also, a huge ‘thank you’ to my oldest friends in ‘sunny’ Colne, who have always made time for a pint or two during my infrequent visits back home and have remained a permanent source of encouragement. I would also like to specially thank my very good friend, Dr. Shaun Leivers, for his knowledge and counsel (mainly after a few pints!) on all matters thesis-, and, indeed, non-thesis(!)-related.

A final massive ‘thank you’ to my amazing girlfriend, Amy Monnington, who has been there to celebrate the good days and has patiently tolerated me on the not-so-good days. Without her to lift my spirits and spur me on, I don’t think I would have lasted the distance (or stayed within the socially acceptable limits of sanity!).

“I can no other answer make but thanks. And thanks, and ever thanks.”

– Twelfth Night, William Shakespeare

*This thesis is dedicated to my parents, Patrick 'Pa' Patten and Angela Barnes.
Thank you both for the countless hours of work you've had to endure and the vast
number of sacrifices (mainly financial!) you've made to get me where I am today.
I just hope I've done you proud in return!*

Love you! x

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Publications

Conference abstracts

Collett, A. and Patten, D. (2012) Could antibiotic-derived membrane vesicles play a role in inflammation during oral antibiotic treatment of an infection? (Poster), 8th *INRA-Rowett Symposium on Gut Microbiology*, P144, Clermont-Ferrand, France

Patten, D. and Collett, A. (2012) *E. coli* C25: Commensal or Pathogen? (Poster), 8th *INRA-Rowett Symposium on Gut Microbiology*, P145, Clermont-Ferrand, France

Manuscripts

Commissioned review article:

Patten, D.A. & Collett, A. (2013) Exploring the immunostimulatory potential of extracellular factors derived from the enteric bacterial microflora, *Microbiology* (In press)

Abbreviations

Abbreviation	Meaning
AMP	Antimicrobial peptide
APC	Antigen presenting cell
BSA	Bovine serum albumin
BT	Bacterial translocation
CD	Crohn's disease
CD14	Cluster of differentiation 14
cDNA	Complementary DNA
CDT	<i>C. difficile</i> binary toxin
cfs	Cell-free supernatant
CFU	Colony forming unit
CO ₂	Carbon dioxide
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EPS	Extracellular polysaccharide
F-actin	Filamentous actin
F-primer	Forward primer
FAE	Follicle-associated epithelial layer
FBS	Foetal bovine serum
gDNA	Genomic DNA
<i>g</i>	Gravitational force
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
h	Hours
HBSS	Hank's buffered salt solution
HD	Human (α) defensin
HEPES	Hydroxyethyl piperazineethanesulfonic acid

HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
IgA	Immunoglobulin A
IL	Interleukin
JAM	Junctional adhesion molecule
LAB	Lactic acid bacteria
LBP	LPS-binding protein
LP	Lipoprotein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M-cell	Microfold cell
MAMP	Microbial-associated molecular pattern
MHC	Major histocompatibility complex
min	Minutes
MLN	Mesenteric lymph node
mRNA	Messenger RNA
MUC2	Mucin-2
MV	Membrane vesicle
NF- κ B	Nuclear factor κ B
NOD	Nucleotide-binding oligomerisation domain
OD	Optical density
ODN	Oligodeoxynucleotides
OM	Outer membrane
PAMP	Pathogen-associated molecular pattern
PC	Paneth cell
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PP	Peyer's patches
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real-time-PCR
R-primer	Reverse primer

RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription-PCR
s	Seconds
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sp.	Subspecies
TBE	Tris/borate/EDTA
TcdA	<i>C. difficile</i> toxin A
TcdB	<i>C. difficile</i> toxin B
TEER	Trans-epithelial electrical resistance
TGF	Transforming growth factor
Th	Helper T-cell
TJ	Tight junction
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloric acid
TSA	Tryptone soy agar
TSB	Tryptone soy broth
UC	Ulcerative colitis
v/v	Volume per volume
w/v	Weight per volume
ZO	Zonula occludin

Chapter 1

Introduction

1.1 Aims

Mammals have co-evolved with indigenous microbial populations, termed *microflora*, which inhabit a wide range of environmental niches on the body, including the expansive lumenal cavity of the intestine. The mammalian intestinal microflora is considered one of, if not *the*, densest microbial populations in the biological world and, consequently, the intestinal epithelial layer represents a key interface which is constantly exposed to these microorganisms. In healthy individuals, the microflora instils a number of physiological and immunological benefits on the host in return for a safe, nutrient-rich environment; however, the microflora also produces a number of microbial-associated molecular patterns (MAMPs) which, like their pathogen-derived contemporaries, possess the ability to stimulate an inflammatory response. Under normal physiological conditions, these microfloral-derived MAMPs are immunologically tolerated, however, they do have the potential to turn malevolent and the enteric flora has been heavily implicated in inflammatory bowel disease.

The main aims of this literature review are three-fold; firstly, to describe the intestinal epithelium and its innate immune defences; secondly, to discuss the enteric microflora, its composition, role in intestinal development and its contribution to inflammatory bowel disease (IBD); finally, to explore the various extracellular products released by bacteria and their potential for eliciting an immune response in the intestine.

1.2 An overview of the intestine

1.2.1 The gastrointestinal tract

The gastrointestinal (GI) tract is a complex collection of organs which are responsible for the mechanical and enzymatic digestion of nutrients, the absorption of nutrients, fluids and electrolytes and the preparation of unabsorbed materials for excretion. The organs which constitute the GI tract can be separated into two groups, those of the alimentary canal and

those outside the alimentary canal, termed the accessory organs, which aid the digestion and ingestion of nutrients. The alimentary canal spans from the mouth to the anus and comprises the mouth, oesophagus, stomach, small intestine, colon, rectum, anal canal and anus. The accessory organs include the liver, the gall bladder, and the pancreas (Colwell *et al.*, 2004).

1.2.2 The intestine

The intestine is an umbrella term for a major part of the alimentary canal which is responsible for the absorption of nutrients, fluids and electrolytes and the excretion of waste products. The intestine consists of the small intestine (the duodenum, the jejunum and the ileum) and the large intestine (the colon, the rectum and the anal canal). The histological features of the intestine are fundamentally the same throughout its length and comprise of four main layers of tissue: the serosa, the muscularis, the submucosa, and the mucosa (Figure 1.1). The serosa is the outermost layer of the intestinal wall and consists of films of connective tissue which contain the intestine within the abdominal cavity. The muscularis is made up of layers of circular and longitudinal smooth muscles which are responsible for the peristaltic movement of the intestinal contents. The submucosal layer consists of connective tissue, nerve fibres and blood and lymph vessels. The innermost layer, the mucosa, consists of three distinct layers; the muscularis mucosae (a thin layer of circular muscle that separates mucosa from the submucosa), the lamina propria (a layer of connective tissue) and the epithelium (which forms a barrier between the contents of the gut and the rest of the body) (Colwell *et al.*, 2004).

The luminal surface of the small intestine is arranged into numerous microscopic finger-like projections (villi) and recesses (crypts) (Figure 1.1) which increase its surface area 10-fold (DeSesso and Jacobson, 2001). The large intestine also contains crypts, which recede into the lamina propria, thus increasing its surface area. Additionally, intestinal epithelial

cells, or enterocytes, also possess thousands of microvilli on their luminal surface which increase the surface area a further 20-fold (DeSesso and Jacobson, 2001). Consequently, the intestine represents the body's largest surface area, with the adult human intestine estimated to cover an area of 100-400 m² (Artis, 2008, Lievin-Le Moal and Servin, 2006, MacDonald and Monteleone, 2005). The large surface area of the intestine aids in absorption, however, paradoxically, it also represents the largest surface area of the body exposed to the environment and its high bacterial load (DeSesso and Jacobson, 2001).

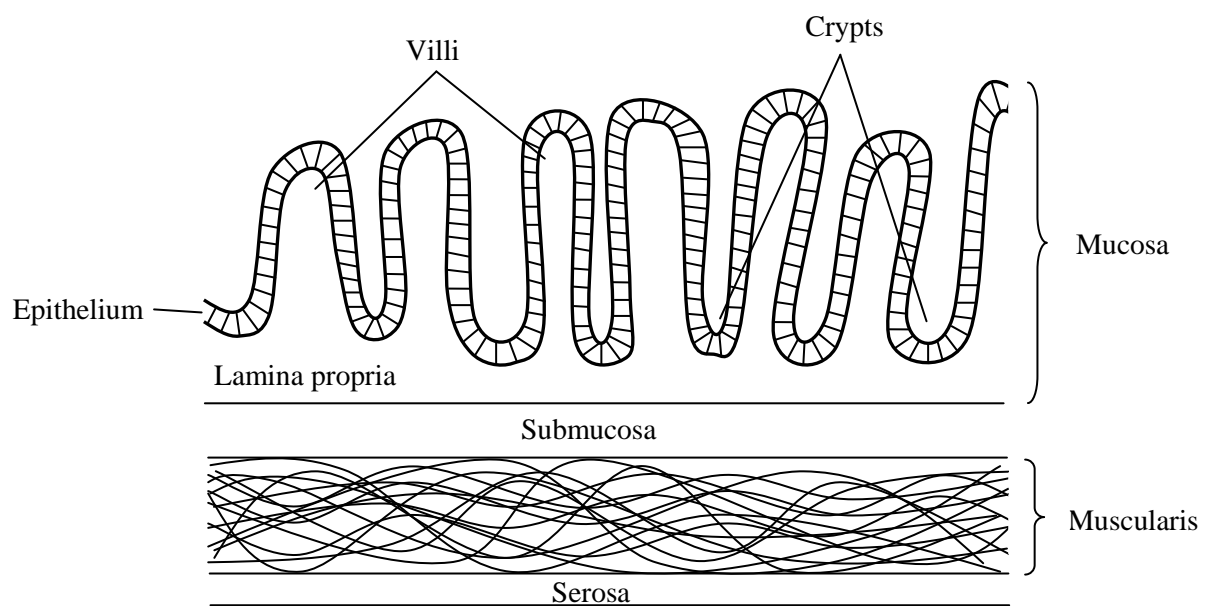


Figure 1.1 – A cross section of the intestinal wall

1.2.3 The epithelial layer

The entire mucosal surface of the GI tract is lined by a one-cell thick (~10 µm) layer of columnar epithelial cells, which acts to separate the luminal contents from the rest of the body (Hooper *et al.*, 2012). The epithelium consists mainly of absorptive (brush border) enterocytes, but also contains a small number of specialised cells, such as mucus-secreting goblet cells (section 1.3.3.2), antimicrobial peptide-producing Paneth cells (section 1.3.3.3) and hormone-releasing enteroendocrine cells (Fuchs and Segre, 2000). Multipotent stem

cells, originating at the villus-crypt interface, mitotically divide, thus producing new cells which subsequently migrate towards the tip of the villus, differentiating into enterocytes, goblet cells or enteroendocrine cells (Fuchs and Segre, 2000). These cells are constantly exposed to the mechanical and enzymatic stresses of the intestinal lumen and, consequently, are sloughed off after 2-3 days and are excreted in faecal matter (Creamer *et al.*, 1961). Alternatively, epithelial stem cells migrate to the base of crypts and form Paneth cells (Fuchs and Segre, 2000). These crypt-sheltered epithelial cells have a much longer turnover period of 18–23 days and are individually removed by phagocytosis (Porter *et al.*, 2002).

1.2.4 Intestinal microflora

Residing in the luminal cavity is a dense population of microorganisms, termed the *microflora*. Estimates suggest that $\sim 10^{14}$ bacteria inhabit the intestine, with 300-500 strains represented (Gill *et al.*, 2006, Guarner and Malagelada, 2003); however, it is worth noting that the intestinal microflora does not solely consist of bacteria, with methanogenic archaea, eukaryotes (yeasts) and viruses (mainly bacteriophages) also present (Lozupone *et al.*, 2012).

The population of the microflora alters throughout the intestine, and is highly dependent on locality. Within the small intestine, the duodenum has a relatively sparse bacterial content (10^4 - 10^5 CFU/ml luminal fluid), due to the low pH of the digesta released from the stomach and the subsequent secretion of pancreatic juices and bile. Conversely, the jejunal microflora reaches levels of 10^5 - 10^7 CFU/ml, and the slower passage of digesta through the ileum allows an even denser bacterial population to flourish (10^7 - 10^8 CFU/ml). Nevertheless, the highest microfloral density is found within the lumen of the colon, with estimates of 10^{10} - 10^{11} CFU/g colonic contents (Ouwehand *et al.*, 2002).

Although the composition of the intestinal microflora is highly variable between individuals (Lozupone *et al.*, 2012), recent metagenomic analyses of human faecal bacteria have given an insight into the phylum- and genus-level composition of the average microflora (Arumugam *et al.*, 2011). The main bacterial phylum represented in faecal microflora is that of *Firmicutes*, which is made up of genera such as ruminococci, clostridia and lactobacilli, and represents ~40 % of the bacterial flora. The next most abundant phylus is the *Bacteroidetes*, (e.g. *Bacteroides* and *Prevotella*) with ~20 % of the bacterial population, followed by *Actinobacteria* (e.g. bifidobacteria and faecalibacteria) with ~8 % and *Proteobacteria* (e.g. *Escherichia/Shigella*) making up ~2 % of the bacterial flora (Arumugam *et al.*, 2011).

Prior to metagenomic studies, traditional bacterial culturing (from intestinal isolates) was utilised to give a rudimentary indication of the regional variation of bacterial genera throughout the different environmental niches of the intestine. The duodenal microflora is highly influenced by the gastric microflora, and so comprises mainly of aciduric facultative anaerobes such as lactobacilli, streptococci, and *Helicobacter pylori* (Ouwehand *et al.*, 2002). In comparison, the jejunal microflora is more diverse, with strains of streptococci, lactobacilli, corynebacteria, *Bacteroides* and *Actinomyces* all identified (Jutesen *et al.*, 1984). Furthermore, the slower passage of the digesta through the ileum means its microflora spans an even wider range of bacterial genera, and both facultative anaerobes, such as, lactobacilli, enterococci and *Enterobacteriaceae*, and obligate anaerobes, such as clostridia and *Bacteroides*, have been cultured from isolates (Jutesen *et al.*, 1984; Ouwehand *et al.*, 2002). Finally, in the colon, the number of strictly anaerobic bacteria out-weighs the number of aerobes or facultative anaerobes by a factor of 100-1000, with genera such as *Bacteroides*, bifidobacteria, clostridia, bacilli and ruminococci making up the vast majority of the bacterial inhabitants (Ouwehand *et al.*, 2002).

1.3 Intestinal immune defences

Due to the chronic immunological challenge posed by the vast microfloral population, a constant low-level of inflammation is hypothesised to be present in the intestine (Macpherson and Harris, 2004; Artis, 2008). However, healthy individuals are able to remain hyporesponsive to the commensal flora and simultaneously retain the ability to mount a rapid and potent effector response to pathogenic microorganisms (Duchmann *et al.*, 1995). To achieve this, the intestinal immune defences are separated into two very distinct, yet inexplicably intertwined, elements; the innate and adaptive immune systems. Adaptive immunity represents the immunological memory and ultimately relies on the generation of a random and highly diverse repertoire of antigen-specific receptors on lymphocytes (T-cell receptors (TCRs) and B-cell receptors (BCRs)) subsequent to antigen challenge (Medzhitov, 2001). Consequently, this system is not designed for immediate response to microbial antigens; therefore a more rudimentary detection mechanism, the innate immune system, exists. The innate immune system is an evolutionarily ancient host defence system that predates the split of the plant and animal kingdoms, and offers a more immediate reaction to infection (Janeway Jr. and Medzhitov, 2002). As with adaptive immunity, the innate system utilises lymphocytes and antigen-binding receptors, however the innate system is germline-encoded and is not reliant on prior antigenic exposure.

The major reaction mechanism employed by the innate system is the inflammatory response. Inflammation is a protective measure utilised by the body to ensure the effective removal of unfavourable stimuli (Takeuchi and Akira, 2010) and is characterised by increased vascular permeability, thus allowing immune cells and serum to infiltrate the site of stimulation (Larsen and Henson, 1983).

1.3.1 Intestinal adaptive immunity

The adaptive immune system is largely represented in the intestine by the gut-associated lymphoid tissues (GALT), which consist of highly organised lymphoid structures, such as Peyer's patches (PPs) and the mesenteric lymph nodes (MLNs) (Forchielli and Walker, 2005). The GALT is the human body's largest collection of lymphoid tissue, and consequently contains ~70 % of the body's total immunocytes (Heel *et al.*, 1997; Forchielli and Walker, 2005).

Peyer's patches are aggregated clusters of sub-epithelial lymphoid follicles located throughout the small intestine (Heel *et al.*, 1997). They consist of a follicle-associated epithelial layer (FAE), a sub-epithelial dome region and the underlying lymphoid follicles themselves (Artis, 2008). The FAE constitutes specialised epithelial cells, called microfold (M)-cells, which lack microvilli and a protective mucus layer (see Section 1.3.3.2), but are so-named due to their broadly folded apical membranes (Mowat, 2003). M-cells constantly sample the luminal contents and deliver any pathogens, or antigens, to the underlying sub-epithelial dome by vesicle transcytosis (Sharma *et al.*, 2009). The sub-epithelial dome consists of antigen-presenting cells (APCs) of the innate immune system (see Section 1.3.2), which capture and process antigens presented by the M-cells and deliver them to the lymphoid follicles (Artis, 2008). The follicles themselves are comprised of a germinal centre surrounded by aggregates of T-cells and B-cells and are separated by interfollicular zones of helper T-cells (see below) (Heel *et al.*, 1997).

MLNs are the largest lymph nodes in the body and act as gatekeepers to the circulatory system (Mowat, 2003). They allow the passage of fully differentiated T-cells and B-cells, from the lamina propria to the circulatory system (where they reside until subsequent antigenic challenges), but prevent commensal-laden APCs from gaining access to the rest of

the body (Macpherson and Uhr, 2004). This produces an effective local immune response without needlessly triggering systemic immunity (Macpherson and Uhr, 2004).

The intestinal wall holds the body's largest collection of T-cells (Heel *et al.*, 1997), which exist within the lamina propria as part of the highly organised lymphoid tissues, such as PPs and MLNs, as freely dispersed moieties within the lamina propria (lamina propria lymphocytes (LPLs) (Davies and Parrott, 1981)) or as integrated epithelial components (intraepithelial lymphocytes (IELs) (Hayday *et al.*, 2001)). Two major functions of T-cells are the regulation of development and activity of other leukocytes (helper T-cells (T_h)), or as cytotoxic effectors (cytotoxic T-cells (T_c)) (Heel *et al.*, 1997). Helper T-cells stimulate macrophages, promote antibody isotype switching (to IgA) in B-cells and activate cytotoxic T-cells (Heel *et al.*, 1997). Cytotoxic T-cells act to suppress IgA production by B-cells and are strongly antagonistic towards APC-processed target cells (Heel *et al.*, 1997).

B-cells of the GALT produce pathogen- or antigen-specific antibodies that cover the mucosal surface and act to agglutinate pathogens, aiding in their phagocytosis (by granulocytes or monocytes) or their excretion in faecal matter (Heel *et al.*, 1997). The predominant antibody in the intestine is immunoglobulin (Ig)A, which usually exists in the proteolysis-resistant dimeric form, IgA2 (Ouwehand *et al.*, 2002). B-cells associated with the human intestine produce 3-5 g IgA/day (MacDonald and Monteleone, 2005), which accounts for ~70 % of body's total antibody production (Macpherson and Uhr, 2004). IgA constitutes 24-74 % of faecal matter and ~50 % of faecal anaerobic bacteria are coated in the antibody (van der Waaij *et al.*, 1996). Unlike the major systemic antibody, IgG, IgA does not provoke an inflammatory response, alternatively it binds antigens and excludes them from the intestinal mucosa (Ouwehand *et al.*, 2002). Also, it has previously been shown in mice that B-cell IgA response against commensal antigens is T-cell independent, whereas induction of IgA by pathogen-derived antigens requires costimulation by antigen-specific T-cells

(Macpherson *et al.*, 2000; Hooper and Gordon, 2001). These mechanisms allow the mucosal immunity to react to autonomously, thus avoiding systemic immune stimulation.

1.3.2 Innate immunity of the intestine

The major functions of the innate immunity are three-fold; the rapid recognition of a diverse range of pathogens and antigens; the subsequent killing of pathogens and the presentation of antigens to the adaptive immunity; and the tolerance of ‘self’ cells and tissues (Medzhitov, 2001, Beutler, 2004). The innate immune system largely relies on monocyte-derived antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs), which subsequently initiate the adaptive system by presenting processed antigens to T-cells, via the class II major histocompatibility complex (MHC) antigen (Beutler, 2004), a mechanism which significantly increases the efficiency of immune stimulation (Banchereau and Steinman, 1998). APCs utilise phagocytosis to engulf microorganisms and initiate a killing pathway which digests them via hydrolytic enzymes, contained within lysosomes (Greenburg and Grinstein, 2002). Subsequently, microbial antigens are directed to MHC components for presentation to adaptive immune cells.

The main function of macrophages is phagocytic killing of pathogenic microorganisms and neutralisation of their associated antigens (Beutler, 2004). However, killing of translocating (see Section 1.4.5) commensal bacteria by macrophages in the lamina propria helps to prevent unnecessary mucosal inflammation and immune response by the adaptive system (Macpherson and Uhr, 2004). Macrophages also release chemokines which attract other myeloid cells, in particular polymorphonuclear phagocytes, such as neutrophils (Beutler, 2004).

DCs, on the other hand, function primarily to signal the presence of pathogens and their antigens, and exhibit diminished killing efficiency when compared to macrophages

(Nagl *et al.*, 2002). DCs constantly sample intestinal lumen by extending intraepithelial dendrites (characteristic elongated cellular projections) to the apical surface. The epithelial integrity is unaffected due to the expression of tight junction (TJ) proteins (see Section 1.3.3.1) in their dendrites which form TJ-like structures with adjacent epithelial cells (Rescigno *et al.*, 2001). DCs help maintain intestinal homeostasis by preferentially triggering non-inflammatory Th2 cell and B-cell IgA responses (Rimoldi *et al.*, 2005).

In addition to professional immunocytes, a number of non-immune components, such as physico-chemical barriers (the epithelium itself, the mucus layer, antimicrobial peptides and the luminal microflora) and epithelial pattern recognition receptors, play a significant role in the innate immunity of the intestine. These are discussed in further detail below.

1.3.3 The intestinal epithelium

1.3.3.1 Tight junctions complete the epithelial barrier

The primary function of the intestinal epithelial layer is to operate as a surface for the dynamic exchange of water, ions and nutrients, however, it also plays an important role in the separation of the luminal contents from the underlying mucosal tissues (Madara, 1989). Intestinal epithelial cells achieve this dual purpose by utilising intercellular bonds, called tight junctions (TJs) which both seal the epithelial layer and facilitate the regulated passage of small molecules (Gonzalez-Mariscal *et al.*, 2003). TJs form a belt-like structure at the apical pole of epithelial cells which, in turn, form a network of close contacts between the membranes of adjacent cells, resulting in a continuous epithelial barrier (Popoff and Geny, 2009). The permeability of TJs is regulated by its association with the intracellular actin cytoskeleton (Nusrat *et al.*, 2000).

TJs consist of a complex arrangement of over 40 proteins (Gonzalez-Mariscal *et al.*, 2003) and the first major breakthrough in deciphering their structure came when Stevenson *et*

al. (1986) identified a conserved intracellular protein, zonula occludin (ZO)-1, in the TJs of hepatocytes. ZO-1 is a member of the membrane-associated guanylate kinase (MAGUK) family and forms a functional link between the TJ and the actin cytoskeleton (Fanning *et al.*, 1998). Subsequently, ZOs-2 and -3 were also found to form a complex with ZO-1 and bind the actin cytoskeleton (Gonzalez-Mariscal *et al.*, 2003). In the *in vitro* characterisation of TJ formation, migration of ZO-1 to the apical pole of the epithelial cell is subsequently followed by the progressive accumulation of associated proteins, which eventually form the belt-like TJs (Gonzalez-Mariscal *et al.*, 2003).

In addition to the submembranous scaffolding of ZO proteins, membrane-bound extracellular proteins are clearly required for the formation of TJs in the paracellular space. The first transmembrane TJ protein to be identified was occludin, which was found to be localised in the TJs of epithelial and endothelial cells (Furuse *et al.*, 1993). The abundance of occludin has been linked to the degree of sealing of TJs, as molecules from adjacent cells form homophilic bonds with each other in the paracellular space (Gonzalez-Mariscal *et al.*, 2003). However, occludin gene double knockout cells were seen to still possess well developed TJs, indicating occludin is not essential to TJ integrity and that other transmembrane proteins play a role in TJ maintenance (Furuse *et al.*, 1998). This led to the further discovery of Claudins-1 and -2 (Furuse *et al.*, 1998) and junctional adhesion molecule (JAM) (Martin-Padura *et al.*, 1998). The cytoplasmic domains of Claudin-family proteins associate with ZOs-1, -2 and -3 while their extracellular loop regions span the paracellular space and interact with claudins from adjacent cells, forming the backbone of TJs (Gonzalez-Mariscal *et al.*, 2003). There is some evidence to suggest that Claudin proteins could also function as paracellular channels for metal ions, as Claudin-16 is selective to Mg^{2+} and Ca^{2+} ions (Gonzalez-Mariscal *et al.*, 2003). JAMs directly contribute to cell-cell adhesion in TJs

with their extracellular immunoglobulin domains forming homophilic interactions with those of neighbouring cells (Popoff and Geny, 2009).

TJs are an obvious target for intestinal disease and number of stimuli can interfere with their integrity, and consequently, the integrity of the epithelium as a whole. Pro-inflammatory cytokines, such as interferon (IFN)- γ , and enteropathogenic bacteria and their toxins have been shown to acutely impede junctional stability (Madara and Stafford, 1989; Capaldo and Nusrat, 2009; MacCallum *et al.*, 2005; Nusrat *et al.*, 2001). Augmented TJ permeability has also been shown in epithelia from chronically damaged intestinal tissues, resulting in exposure of the underlying tissues to the luminal contents (Capaldo and Nusrat, 2009).

1.3.3.2 Mucus – a frontline defence

Supporting the one-cell thick intestinal epithelial barrier is a gel-like layer of mucus (Deplancke and Gaskins, 2001). This mucus layer forms a protective coating over the epithelium, shielding it against chemical, enzymatic and mechanical damage and, very importantly, preventing luminal bacteria from gaining direct access to the epithelial layer (Smirnova *et al.*, 2003). Interestingly, the mucus layers of the small intestine and colon are quite contrasting in their physical properties. The mucus layer of the small intestine is a thin, permeable film interrupted by the mucus-free Peyer's patches (see Section 1.3.1) (Deplancke and Gaskins, 2001). This is tailored to the physiological role played by the small intestine, in which large amounts of water and nutrients are absorbed. Also, the relatively low bacterial density in the small intestine means that the permeability of the mucus layer is of little consequence (Johansson *et al.*, 2011). Conversely, the mucus of the densely inhabited colon is a continuous layer of increasing thickness (from the ascending colon to the rectum) which consists of an adherent inner layer and a non-adhered outer layer (Johansson *et al.*, 2008).

The inner layer is tightly associated with the epithelium and has a very dense framework, thus presenting a sterile, impermeable barrier to luminal bacteria (Johansson *et al.*, 2011). Conversely, the outer layer presents the ideal habitat for bacteria (Kirjavainen *et al.*, 1998), as it offers an easily accessible source of carbohydrates, peptides and exogenous nutrients (concentrated within the mucus) (Deplancke and Gaskins, 2001). However, the loose consistency of the outer layer which allows the adherence, encapsulation and habitation of commensal bacteria also results in a high turnover rate and the efficient extraction of invading pathogenic bacteria and waste matter (Kirjavainen *et al.*, 1998).

The intestinal mucus is composed of heterogeneous, highly glycosylated proteins called mucins, which are produced and secreted from the apical surface of a specialised sub-type of epithelial cells, termed goblet cells (Smirnova *et al.*, 2003). Goblet cells are distributed throughout the intestinal epithelial lining, in varying numbers, in a site-dependent manner, with the highest density residing in the rectal-terminus of the colon (Deplancke and Gaskins, 2001). Mucin-2 (MUC2) is the major mucin and primary structural constituent of the colonic mucus and is found in both the inner and outer layers (Johansson *et al.*, 2008). MUC2 plays a major role in epithelial homeostasis, as MUC2-deficient mice exhibit increased proliferation and decreased apoptosis of intestinal epithelial cells, leading to the formation of intestinal tumours (Velcich *et al.*, 2002). MUC2-deficient mice also lack the firm inner layer of mucus, allowing the luminal bacteria direct contact with the epithelial layer, leading to increased bacterial translocation (see Section 1.4.5) and augmented epithelial inflammation (Johansson *et al.*, 2008).

1.3.3.3 Paneth cells and antimicrobial peptides

Paneth cells (PCs) are pyramidally-shaped granulocytic cells that are found at the base of epithelial crypts of the small intestine, with each crypt containing, on average, 5-15 PCs

(Ouellette and Selsted, 1996; Porter *et al.*, 2002). Epithelial stem cells, originating from the villus-crypt interface, migrate to the base of crypts and differentiate into PCs, which, upon maturation, produce intracellular apically-associated granules (Porter *et al.*, 2002). PCs secrete immunoglobulin (Ig)A and PAP (pancreatitis-associated protein), which act to aggregate bacteria and promote their binding to host phagocytes, thus aiding the management of the microfloral population and the maintenance of sterility in crypts, especially around the vulnerable epithelial stem cell zones (Porter *et al.*, 2002; Elphick and Mahida, 2005). However, the primary function of PCs is to release their cytoplasmic granule stores (degranulation) in response to acute stimulation by bacteria and their associated antigens, and host inflammatory cytokines, such as tumour necrosis factor (TNF)- α and IFN- α (Porter *et al.*, 2002).

The granules of PCs contain antimicrobial peptides (AMPs), such as phospholipidase A2 (PLA2), lysozyme and defensins, which are all effective against a wide range of bacteria, fungi, protozoa and enveloped viruses (Ouellette and Selsted, 1996). Defensins, in particular α -defensins, such as human defensin (HD)-5 and HD-6, are the major AMP representatives in the intestine (Ouellette and Selsted, 1996), with crypt concentrations capable of reaching in excess of 10 mg/ml (Ganz, 2003). Cationic and amphiphilic by nature, defensins electrostatically bind the negatively-charged surface groups of target organisms and subsequently cause permeabilisation of the membrane by their insertion (Selsted and Ouellette, 2005). Loss of membrane integrity leads to the cessation of RNA, DNA and protein synthesis, and eventually results in the death of the target organism. In addition to their direct antimicrobial activity, defensins are capable of inducing the adaptive immune system as a result of their chemotactic attraction of monocytes, T-lymphocytes and immature dendritic cells (Ganz, 2003; Selsted and Ouellette, 2005).

1.3.4 Pattern recognition receptors

Pattern recognition receptors (PRRs) are germline-encoded sensory molecules, constitutively expressed in all mammalian cell types, which represent a key component of the innate immune system (Akira *et al.*, 2006). PRRs recognise a range of highly conserved microbial moieties, termed pathogen-associated molecular patterns (PAMPs). Mammalian PRRs have evolved to recognise PAMP motifs which are essential to microbial viability, thus significantly lowering the possible emergence of immune-evading pathogenic mutants (Medzhitov, 2001). However, the ability of PRRs to recognise these microbial products is not just limited to pathogens, and so a less used, but more accurate, form of terminology might be MAMPs (microbial-associated molecular patterns) (Medzhitov, 2001).

Two major types of PRRs are Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain (NOD) receptors. TLRs and NOD receptors are both constitutively expressed on a range of intestine-associated immune cells, such as macrophages (Akira *et al.*, 2006), dendritic cells (Iwasaki and Medzhitov, 2004), B-cells and T-cells (Hornung *et al.*, 2002), and are even expressed in non-professional immune cells, such as intestinal epithelial cells (Furrie *et al.*, 2005; Gribar *et al.*, 2008; Bannon, 2008).

1.3.4.1 Toll-like receptors

The Toll family of PRRs is conserved in the innate immune systems of a vast range of multicellular eukaryotes, from the nematode worm (*Caenorhabditis elegans*), to higher order mammals, such as humans (Akira *et al.*, 2006). The founding member of the Toll family, the *Toll* gene product in *Drosophila*, was first identified as an essential protein in the fly's embryonic development (Anderson *et al.*, 1985; Hashimoto *et al.*, 1988). However, Lemaitre *et al.* (1996) discovered that the transmembrane protein also plays a central role in the fruit fly's antifungal immunity. Their study showed that Toll-defective mutants demonstrated a

Pattern recognition receptor (PRR)	Location(s)	Ligand(s)	Source(s)
TLR-2	Plasma membrane	Peptidoglycan Phospholipomannan Haemagglutinin protein	Bacteria Fungi Measles virus
TLR-2/TLR-1	Plasma membrane	Lipoprotein Triacyl lipopeptides	Bacteria Gram-negative bacteria
TLR-2/ TLR-6	Plasma membrane	Zymosan Diacyl lipopeptides Lipoteichoic acid	Fungi Mycobacteria Gram-positive bacteria
TLR-3	Endosomal membrane	dsRNA	Viruses
TLR-4	Plasma membrane	Lipopolysaccharide Mannan	Gram-negative bacteria Fungi
TLR-5	Plasma membrane	Flagellin	Bacteria
TLR-7	Endosomal membrane	ssRNA	Viruses
TLR-8	Endosomal membrane	ssRNA	Viruses
TLR-9	Plasma & endosomal membranes	CpG-DNA	Bacteria Viruses Protozoa
TLR-10	Endosomal membrane	Unknown	Unknown

Table 1.1 – Human TLRs and their known agonists

significantly reduced survival rate after infection with the fungal pathogen, *Aspergillus fumigatus* (Lemaitre *et al.*, 1996). Just 12 months following the discovery of the Toll protein, a human homologue, now termed ‘Toll-like receptor (TLR)-4’, was identified and demonstrated the ability to induce the expression of several proinflammatory cytokines including interleukin (IL)-1 and IL-8 (Medzhitov *et al.*, 1997). Since then, a set of 9 further proteins, structurally related to TLR-4, have been identified in humans and have been shown to confer responsiveness to a wide range of MAMPs (see Table 1.1).

TLRs are type I integral membrane glycoproteins which consist of 3 distinct domains (Botos *et al.*, 2011); a MAMP-binding extracellular domain, which contains a variable number (dependent on TLR-type) of leucine-rich repeats (LRRs) (Bell *et al.*, 2003); a transmembrane domain, which spans the host cell membrane, thus holding the receptor in place; and a cytoplasmic signalling domain, which is homologous to that of IL-1R, and consequently named the Toll/IL-1R homology (TIR) domain. The TIR domain is responsible for the intracellular transmission of the stimulatory signal (Akira *et al.*, 2006).

Binding of TLRs, by their respective agonist(s), triggers activation of intracellular signalling cascades which ultimately result in the induction of genes involved in antimicrobial defence, such as those encoding proinflammatory cytokines (Figure 1.2). Subsequent to ligand binding, TLRs dimerise in the host cell membrane and undergo the appropriate conformational changes required for the recruitment of TIR domain-containing adaptor protein (TIRAP). The TIR region of the adaptor molecule binds to its counterpart in the TLR receptor by homophilic interactions. Next, myeloid differentiation factor 88 (MyD88), an adaptor molecule critical for intracellular signalling from all TLRs (except TLR-3), binds to TIRAP. This complex subsequently recruits IL-1R-associated kinases (IRAKs)-1 and -4, resulting in IRAK-4 activation. Activated IRAK-4 phosphorylates MyD88-associated IRAK-1, thus allowing it to further combine with the ubiquitin protein ligase, tumour necrosis factor receptor (TNFR)-associated factor (TRAF)-6. With the assistance of a ubiquitination enzyme complex, TRAF-6 catalyses the formation of a polyubiquitin chain both on itself and on I κ B kinase (IKK)- γ /NF- κ B essential modulator (NEMO). The complex of transforming growth factor (TGF)- β -activated kinase (TAK)-1 and its binding proteins (TBP-1, -2 and -3) are also recruited to TRAF-6 and TAK-1 phosphorylates IKK- β . IKK- β , in turn, phosphorylates I κ B, thus degrading the I κ B/nuclear factor (NF) κ B complex and freeing NF- κ B to translocate into the nucleus and induce the

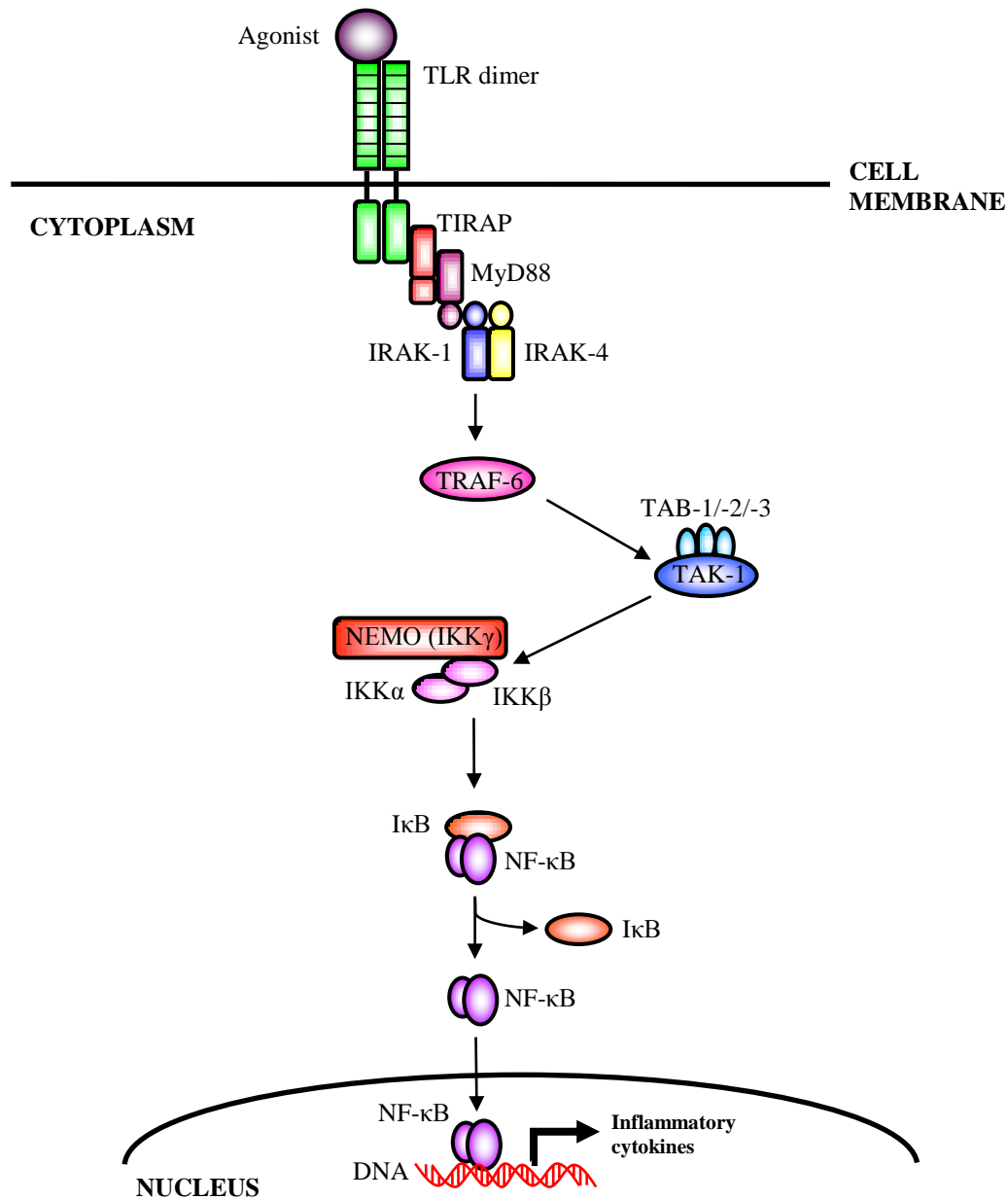


Figure 1.2 - TLR signalling pathway

transcriptional expression of proinflammatory cytokines (Aderem and Ulevitch, 2000; Akira *et al.*, 2006).

The induction of proinflammatory cytokine expression by TLR-mediated pathways must undoubtedly be regulated in a highly stringent manner, as excessive release will inevitably lead to severe systemic inflammation and, ultimately, death (Takeda and Akira, 2005). One of the major regulatory mechanisms is antigenic tolerance. Lotz *et al.* (2006)

demonstrated that antigenic tolerance is an acquired phenomenon and not an inherent feature instilled during foetal development. Their results showed that foetal, neonatal and adult intestinal epithelial cells (IECs) all constitutively expressed the LPS receptor complex, TLR-4/MD-2, but found only foetal cells were responsive to LPS (Lotz *et al.*, 2006). Furthermore, they showed that tolerance to LPS is acquired after exposure to the antigen during passage through the birth canal (individuals born under caesarean section did not exhibit tolerance) and is paralleled by the spontaneous activation of IECs (as represented by up-regulation of macrophage inflammatory protein (MIP)-2 mRNA) (Lotz *et al.*, 2006). The mechanistic specifics of antigenic tolerance are, as yet, unknown; however, it is thought that anti-inflammatory cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)- β are involved (Medzhitov, 2001).

Another key regulatory mechanism is the expression of inhibitory proteins, such as Toll-interacting protein (Tollip). Tollip was originally identified as a key intermediate protein in IL-1 signalling (Burns *et al.*, 2000), and it was subsequently hypothesised that the significant homology between the IL-1R and TIR cytoplasmic domains would result in Tollip also interacting with the TLR signalling pathway (Zhang and Ghosh, 2002). The study undertaken by Zhang and Ghosh (2002) demonstrated that Tollip does in fact interact with the TLR signalling pathway, inhibiting the TLR-2 and TLR-4-induced NF- κ B activation by means of binding to, and inhibiting the kinase activity of, IRAK. Additional suppressor proteins, such as suppressor of cytokine signalling (SOCS)-1 and the macrophage IL-1R-associated kinase (IRAK)-M, also limit cytokine release in response to TLR agonists, as both SOCS-1- and IRAK-M-deficient mice show significantly increased cytokine expression to bacterial infection (Kobayashi *et al.*, 2002; Nakagawa *et al.*, 2002).

As detailed above, TLRs are undoubtedly inflammatory mediators involved in host defence; however, there is some evidence to suggest that TLRs also play a part in

autoimmunity and sterile inflammation (inflammation caused by molecules of dying host cells or products of tissue injury) (Kanzler *et al.*, 2007). An additional, non-immune, function has also been suggested, as, following stimulation by commensal bacteria, TLRs are considered responsible for the induction of factors involved in processes such as cytoprotection, wound healing and angiogenesis (Rakoff-Nahoum *et al.*, 2004).

1.3.4.2 Cytoplasmic pattern recognition receptors – NOD receptors

Intracellular pathogens, which could evade recognition by TLRs are detected by TLR-independent, cytoplasmic PRRs, such as nucleotide-binding oligomerisation domain (NOD) receptors (Akira *et al.*, 2006).

NOD receptors, NOD-1 and NOD-2, belong to the NOD leucine-rich receptor (LRR) family (the NLR family) and are homologous to the evolutionarily ancient plant disease resistance proteins (Abreu *et al.*, 2005). NOD-1 consists of 3 distinct domains, the C-terminal LRR domain, a central NOD domain and an N-terminal caspase recruitment domain (CARD). NOD-2 was identified through its structural similarity to NOD-1, however, its N-terminus contains 2 CARDS (Takeda and Akira, 2005). NOD-1 plays an important role in the detection of Gram-negative intracellular pathogens, as it recognises γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP), a peptidoglycan derivative found exclusively in Gram-negative bacteria (Girardin *et al.*, 2003a; Chamaillard *et al.*, 2003). Conversely, NOD-2 exhibits a more universal recognition ability, binding to muramyl dipeptide (MDP), the minimal bioactive peptidoglycan motif found in both Gram-positive and Gram-negative bacteria (Girardin *et al.*, 2003b; Inohara *et al.*, 2003). Ligand binding to NOD-1 or NOD-2 leads to oligomerisation of the receptors, which induces the recruitment of the serine/threonine kinase Rip2/RICK (Takeda and Akira, 2005). NOD-receptor-bound Rip2/RICK subsequently activates the NF- κ B-mediated expression of proinflammatory

cytokines (Akira *et al.*, 2006; Masumoto *et al.*, 2006).

1.3.5 Cytokines and chemokines

Epithelial PRRs which detect and respond to enteric bacteria (and their associated antigens) necessitate a specific transduction mechanism to relay the stimulatory signal to the host's immune system. This is achieved through expression of a comprehensive range of proteinaceous signal molecules, termed cytokines, which regulate the function of intestine-associated immune cells found in the underlying mucosal tissues.

Cytokines are a group of small, non-structural proteins produced by nucleated cells, whose primary function is to mediate the host response to environmental stresses, such as UV light, heat shock, hyperosmolarity, injury, infection and disease (Dinarello, 2000). There are two main types of cytokines; proinflammatory cytokines, which promote the host inflammatory response; and anti-inflammatory cytokines, which suppress the actions of their proinflammatory counterparts (Dinarello, 2000). A delicate balance between the two is required for the maintenance of homeostasis in the host. During infection, there is a significant increase in proinflammatory cytokines, however, they do not have any direct effect on the invading pathogen themselves, but rather divert blood flow and attract immune cells to the area of stimulation and increase the influx of professional immune cells (Beutler, 2004).

Chemotactic cytokines, or chemokines, are a subset of cytokines which are responsible for the attraction of leukocytes, from circulation, to stress-stimulated tissues (Luster, 1998). Chemokines are subdivided and classified by the relative position of their cysteine residues. In α -chemokines, an amino acid (X) separates the cysteine (C) residues, giving the 'CXC' sequence, however, in β -chemokines the cysteine are adjacent to one another, giving the 'CC' sequence (Luster, 1998). Primarily, α - and β -chemokines are

characterised by their different chemotactic activities, α -chemokines are chemotactic for neutrophils and lymphocytes, whereas β -chemokines attract monocytes, eosinophils, basophils and lymphocytes with variable selectivity (Luster, 1998).

Epithelial-derived cytokines and chemokines play a significant physiological role in the extent and length of the immune response to bacterial (both pathogenic and commensal) stimulation (Schuerer-Maly *et al.*, 1994; Haller *et al.*, 2000; Bahrami *et al.*, 2010).

1.3.5.1 Promoting inflammation – interleukin-8

Interleukin (IL)-8 is an highly potent α -chemokine (CXC) which is responsible for the attraction and activation of neutrophils to the site of stimulated release (Baggiolini *et al.*, 1989). Also, a secondary chemoattractant activity to basophils (Oppenheim *et al.*, 1991) and a physiological role in the stimulation of angiogenesis (Koch *et al.*, 1992) have been suggested. IL-8 is synthesised by a wide variety of cell types, such as mononuclear cells (Yoshimura *et al.*, 1987), fibroblasts (Larsen *et al.*, 1989), keratinocytes (Larsen *et al.*, 1989), endothelial cells (Strieter *et al.*, 1989), hepatocytes (Thornton *et al.*, 1990) and intestinal epithelial cells (IECs) (Lammers *et al.*, 1994; Warhurst *et al.*, 1998; Zhang *et al.*, 2005). In IECs, IL-8 release is significantly increased in response to a range of stimuli, such as primary mediators (IL-1 and TNF) (Lammers *et al.*, 1994), bacterial entry (Eckmann *et al.*, 1993b) and bacterial antigens, such as LPS (Schuerer-Maly *et al.*, 1994).

Despite its important role in the protective inflammatory response, over-expression of IL-8 can have detrimental effects on the body. Excessive production of IL-8 in the intestine, has been linked to the pathophysiology of inflammatory bowel disease (Banks *et al.*, 2003). Elsewhere in the body, IL-8 over-production has been implicated in the aetiology of autoimmune conditions, such as psoriasis and rheumatoid arthritis (Graves and Jiang, 1995).

Therefore, the proinflammatory pathways must be regulated by anti-inflammatory cytokines, such as IL-10.

1.3.5.2 Suppressing the inflammatory response – interleukin-10

Interleukin (IL)-10 was originally named ‘cytokine synthesis inhibitory factor’, on account of its inhibitory action against cytokine production in T-helper (Th)1 cells. Subsequently, numerous other immune functions of the chemokine were uncovered, leading to its name re-evaluation (Moore *et al.*, 2001). IL-10 is very potent anti-inflammatory mediator, and is a key ameliorator of excessive inflammation in response to pathogenic attack, with the strength of the regulatory IL-10 response reflecting the strength of the preceding inflammatory response (Williams *et al.*, 2004; Couper *et al.*, 2008). IL-10 is mainly secreted by professional immune cells, such as T-cells, B-cells, macrophages and dendritic cells, however, non-immune cells, such as epithelial cells, have also shown expression (Mosser and Zhang, 2008; Bahrami *et al.*, 2010; Gao *et al.*, 2012). The main biological function of IL-10 is exerted on innate immune cells, such as dendritic cells and macrophages, acting as a potent inhibitor of antigen presentation (Mosser and Zhang, 2008) and actively destabilising the mRNA of proinflammatory chemokines, thus preventing their expression (Moore *et al.*, 2001). Additionally, IL-10 prevents the mRNA degradation of other anti-inflammatory mediators, thus enhancing their expression (Moore *et al.*, 2001).

IL-10 undoubtedly plays a significant role in mucosal immune regulation as IL-10-deficient mice develop enterocolitis (Moore *et al.*, 2001). Further to this, IL-10 demonstrates prevention of inflammation and mucosal ulceration in murine colitis models (Steidler *et al.*, 2000; de Moreno de LeBlanc *et al.*, 2011).

1.4 Intestinal microflora

1.4.1 Acquisition of commensal bacteria

In utero, the foetal intestine is sterile and bathed in amniotic fluid (Fanaro *et al.*, 2003). However, prior to birth, the amniotic fluid is drained and, during the consequential passage through the birth canal, the intestine is colonised by microorganisms from the mother's vaginal flora and the surrounding environment (Ouwehand *et al.*, 2002). Bacteria appear in the faeces of a newborn infant within the first few hours after birth and the enteric commensal microflora progressively flourishes in the first week of life outside the uterus (Ouwehand *et al.*, 2002). The neonatal intestine is firstly colonised by facultative anaerobes, such as streptococci, staphylococci and enterobacteriaceae (specifically *E. coli*) (Hooper, 2004). These early colonisers consume the oxygen within the (initially aerobic) intestinal lumen, reducing the oxidation-reduction potential, thus providing more favourable conditions for stricter anaerobes, such as lactobacilli, bifidobacteria and *Bacteroides* species (Fanaro *et al.*, 2003). Upon introduction of solid foods to the diet, the commensal microflora undergoes significant shifts in its composition, and by the age of 2 years old the microflora begins to reflect that of an adult (Ouwehand *et al.*, 2002). Interestingly, babies delivered by caesarean section exhibit delayed microfloral development in comparison to those born vaginally and bacterial compositional differences are evident between babies delivered by the two birth methods (Ouwehand *et al.*, 2002; Fanaro *et al.*, 2003).

1.4.2 Roles and functions of the intestinal microflora

The colonisation of the host intestine by the indigenous microbial population is the result of millions of years of co-evolution and, consequently, a highly complex symbiotic relationship has emerged (Hooper and Gordon, 2001; Artis, 2008). The intestine provides a hospitable, and relatively pathogen-free, environment for the bacteria, which is both temperature-stable

and nutrient-rich (Artis, 2008). In return, the commensal microflora performs two main functions which benefit the host; firstly, it provides an additional physical barrier between potential pathogens and the epithelial layer; secondly, and more importantly, it aids the overall development of the intestine.

Studies utilising germfree mice have given a fascinating insight into the role the microflora plays in the morphological, immunological and metabolic development of the intestine. Germfree (gnotobiotic) mice are born by sterile caesarean section and are reared in sterile conditions, resulting in a complete lack of colonisation of the body by microorganisms (Gordon and Pesti, 1971). The word *gnotobiotic* is derived from the Greek words ‘gnotos’ and ‘biota’, and literally translates to ‘known flora’ (Gordon and Pesti, 1971), and so the term can also refer to ex-germfree mice colonised with a known single bacterium, or combination of bacteria. A number of distinct phenotypic differences are observed in germfree mice when compared to those conventionally raised.

Morphologically, the intestinal wall of germfree mice varies quite considerably from that of their conventionally raised counterparts, with severe differences such as; hypoplasia of intestinal villi, including low levels of vascularisation (Syed *et al.*, 1970, Hooper, 2004); shallowing of crypts in the ileum (Syed *et al.*, 1970); and significantly reduced epithelial turnover (Savage *et al.*, 1981). An absence of microflora also results in the accumulation and inspissation of epithelial mucus, as the degradation and clearance processes are significantly reduced (Neish, 2002).

Colonisation of the intestine by non-pathogenic bacteria presents fierce competition for environmental niches and nutrients, thus excluding the majority of potentially pathogenic bacteria (Farthing, 2004). Additionally, the microflora plays an essential role in the maturation of the intestinal immunity as, at birth, the immune system is extremely naïve, as can be demonstrated in germfree animals. When contrasted with conventionally reared

animals, germfree animals exhibit significantly reduced production of antimicrobial peptides due to lower numbers of Paneth cells (Guarner and Malagelada, 2003). Additionally, their adaptive immunity displays considerably fewer intraepithelial lymphocytes (IELs) (Neish, 2002), smaller Peyer's patches due to slower development of lymphoid follicles (Forchielli and Walker, 2005) and lower levels of mucosal IgA resulting from diminished numbers of antibody-producing B-cells (Hooper *et al.*, 2012). Consequently, circulatory levels of antibodies are also much lower in germfree mice, therefore oral tolerance of antigens only lasts a matter of days, whereas in conventional mice it subsists for months (Guarner and Malagelada, 2003). However, despite the extreme disparities, upon exposure to normal commensal microflora, ex-germfree animals are able to develop an immunity comparable to that of conventionally-raised animals (Ouwehand *et al.*, 2002). The culmination of the morphological and immunological changes (due to the lack of microflora) seen in germfree animals results in a luminal surface that is significantly less efficient in absorption than its conventionally-raised counterpart and is considerably more likely to succumb to pathogenic attack.

The term *commensal* originates from the Latin 'commensalis', which means 'at the table together', and indeed, the microflora represents a metabolically active entity that aids host nutrition (Hooper and Gordon, 2001). The microflora is responsible for the enzymatic degradation of substances that would otherwise be indigestible to the host, for example complex carbohydrates, such as cellulose, resistant starch, inulin and xylans (Hooper, 2004, Tremaroli and Bäckhed, 2012). These complex carbohydrates are fermented by the microflora, producing the short chain fatty acids (SCFAs), such as butyrate, propionate and acetate (Tremaroli and Bäckhed, 2012). Butyrate is utilised locally as an energy substrate for epithelial cells and also plays a role in epithelial integrity and homeostasis (Nicholson *et al.*,

2012). Conversely, propionate and acetate are transported to distal organs (Nicholson *et al.*, 2012).

The intestinal flora also assists host nutrition via the synthesis of vitamins, such as vitamin K, and the absorption of calcium, magnesium and iron (Neish, 2002, Guarner and Malagelada, 2003). The enteric microflora also triggers the storage of fat, a trait highly beneficial to the sporadic diet of ancient humans, but one that has become largely redundant and detrimental in a modern society of large portions and high-calorie food, thus predisposing to the prevalence of obesity (Bäckhed *et al.*, 2004). Furthermore, microfloral shifts have been documented in obese mice and humans. Obese individuals exhibit significantly increased levels of Firmicutes and ~ 50 % fewer Bacteroidetes, resulting in a microflora more adept at releasing calories from food, which are thus absorbed by the host (Ley *et al.*, 2006; Turnbaugh *et al.*, 2006).

1.4.3 Probiotics

The term *probiotic*, is derived from the Greek words meaning ‘for life’, and was originally coined to describe ‘substances secreted by one microorganism which stimulate the growth of another’ (Schrezenmeir and de Vrese, 2001). Subsequently, the *probiotic* idiom was often used in the context of bacteria-containing animal feeds, and the definition subtly evolved to ‘organisms and substances which contribute to intestinal microbial balance’. However, this was significantly revised due to the implication that ‘substances’ could be referring to antibiotics (Fooks *et al.*, 1999). Also, the health benefits of probiotic bacteria were found to supersede modifications of the intestinal microflora (Blum *et al.*, 2002); thus, current definitions describe probiotics as ‘live microorganisms which, when orally administered in adequate amounts, confer a health benefit on the host’ (Wohlgemuth *et al.*, 2010).

One of the major health benefits associated with probiotics is increased protection against pathogen attack. Probiotics often promote enhanced expression and redistribution of TJ proteins, thus increasing epithelial barrier integrity and limiting the passage of potential pathogens to the underlying mucosal tissues (O'Flaherty and Klaenhammer, 2010). Also, probiotic bacteria exploit the same epithelial binding site receptors as pathogenic bacteria and therefore present fierce competition for both environmental niches and nutrients, which often results in the competitive exclusion of the invading pathogen, from the intestine (Fooks *et al.*, 1999, Vinderola *et al.*, 2005). Another mechanism often utilised against pathogens by probiotic bacteria is the production of antibacterial compounds, such as bactericidal proteins (bacteriocins) and lactic acid (Wohlgemuth *et al.*, 2010). The host's immune responses are also manipulated by probiotic bacteria in order to ensure pathogenic clearance, with immune functions, such as augmented defensin secretion by Paneth cells, increased production of secretory IgA and increased natural killer (NK) cell activity, previously observed (Erickson and Hubbard, 2000; Wohlgemuth *et al.*, 2010).

Within the intestine, health benefits imparted by probiotics include improved digestion of lactose in intolerant hosts, prevention of food allergies, lessening of diarrhoeal severity and reduction in symptoms of irritable bowel syndrome (IBS) (Galdeano *et al.*, 2007; O'Flaherty and Klaenhammer, 2010). In addition to the local positive effects of probiotics in the intestine, more systemic benefits have also been characterised, with probiotics acting to lower blood cholesterol, decrease the severity of bronchial infections in infants and confer anticarcinogenic activity at sites distal to the intestine (O'Flaherty and Klaenhammer, 2010; Wohlgemuth *et al.*, 2010). It has also been suggested that probiotic bacteria could represent a novel delivery method for heterologous proteins of vaccinal, medical or technological interest (del Carmen *et al.*, 2011).

1.4.4 Prebiotics and synbiotics

The concept of *prebiotics* was originally conceived by Gibson and Roberfroid (1995), who defined them as ‘non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth, and/or the metabolic activity, of a limited number of colonic bacteria which subsequently induce favourable lumenal or systemic affects’. They also suggested the notion of combining probiotics and prebiotics to work synergistically in the growth promotion of beneficial bacteria in the host colon, forming *synbiotics* (Gibson and Roberfroid, 1995).

1.4.5 Breaching the epithelial layer – bacterial translocation

Bacterial translocation (BT) can be defined as ‘the passage of both viable and non-viable microbes and microbial products across an anatomically intact intestinal barrier’ (Alexander *et al.*, 1990). Further to this, there are two major pathways of translocation; transcellular i.e. through the cells of the epithelium; and paracellular, in which microbes pass through the intercellular tight junctions (Balzan *et al.*, 2007). BT in the intestine is traditionally associated with the presence of predisposing factors, such as bacterial overgrowth, damage to the epithelial layer or the infiltration of pathogenic bacteria (Balzan *et al.*, 2007; Gatt *et al.*, 2007). However, Berg (1995) suggested that indigenous bacteria constitutively translocate transcellularly (thus not affecting epithelial integrity) from the intestinal lumen of healthy, immunocompetent individuals, but are killed en route or *in situ* once they reach the lymphoid organs. Furthermore, Lichtman *et al.* (2001) suggested that BT is required to allow the GALT to generate immunocompetent cells. There is some, albeit limited, evidence to support these theories, with a range of non-pathogenic commensal bacteria exhibiting (low) levels of BT and immune stimulation *in vitro*, in intestinal epithelial cell lines (Reddy *et al.*, 2007; Ohkusa *et al.*, 2009). Additionally, the translocation of the commensal microflora, and consequent

stimulation of the host immune system, has been heavily implicated in the pathophysiology of inflammatory bowel disease (see Section 1.4.6).

1.4.6 Mutinous microflora – inflammatory bowel disease

Inflammatory bowel disease (IBD) is an idiopathic chronic disorder, occurring in immunocompetent individuals (Cobrin and Abreu, 2005), which affects approximately 1 in every 350 people in the UK (NHS, 2011). The characteristic symptoms of IBD, such as abdominal pain, diarrhoea, rectal bleeding, malaise and weight loss (Strober *et al.*, 2007, Reiff and Kelly, 2010), all arise from a non-infectious, cytokine-driven inflammation of the intestine (Cobrin and Abreu, 2005; Strober *et al.*, 2007). The chronic inflammation associated with IBD has also been associated with an increased incidence of colorectal cancer (Atreya and Neurath, 2010).

IBD exists in two major forms, Crohn's disease (CD) and ulcerative colitis (UC). CD was first observed by the German surgeon, Wilhelm Fabry in the early 17th century (Baumgart and Carding, 2007), however, it remained largely unknown until the condition was later described by, and subsequently named after, the American gastroenterologist, Burril B. Crohn in the early 1930s (Crohn *et al.*, 1932). CD is characterised by transmural intestinal inflammation (Strober *et al.*, 2007), and non-necrotising granuloma formation resulting from the aggregation of macrophages (Xavier and Podolsky, 2007). In more severe cases, the development of obstructing strictures of the intestine or inflammation of connections between the intestine and other organs can occur (Strober *et al.*, 2007). CD may affect any site of the GI tract, but mainly occurs in the ileum and colon (Xavier and Podolsky, 2007). Conversely, ulcerative colitis was first described in the mid-19th century by the British physician Sir Samuel Wilks, and was thus named due to the severe inflammation and extensive superficial mucosal ulceration observed (Wilks, 1859). UC occurs exclusively in the distal ileum and

colon (Bene *et al.*, 2011) and is histopathologically characterised by the excessive presence of neutrophils in the lamina propria and crypts, resulting in micro-abscesses (Xavier and Podolsky, 2007).

1.4.6.1 Pathophysiology of IBD

Under normal physiological conditions, healthy individuals remain systemically impassive to the wide array of food and microfloral antigens within the intestine (Bene *et al.*, 2011); however, in IBD this tolerance is lost and sufferers of the disease exhibit serological and T-cell responses to commensal-derived antigens (Sartor, 2008). Excessive immune reaction to the microflora leads to the chronic inflammation of the intestinal mucosa classically associated with IBD (Bene *et al.*, 2011). The exact aetiological mechanisms of IBD are still uncertain; however intense research in this area has uncovered a number of contributing factors, such as genetic susceptibility, the commensal microflora itself, immune abnormalities and environmental influences.

In the 1930s, studies reported a familial aggregation of IBD (Baumgart and Carding, 2007) and subsequent investigations in to genetic susceptibility utilised twin concordance studies (Tysk *et al.*, 1988; Thompson *et al.*, 1996). Monozygotic (identical) twins demonstrated a pooled systemic concordance of 37.3 % for CD and 10 % for UC, whereas dizygotic (non-identical) twins showed concordances of 7 % and 3 % for CD and UC, respectively (Baumgart and Carding, 2007). This indicated that genetic predisposition was a larger contributor to CD susceptibility than in UC. Genome-wide searches have identified over 70 genes within loci previously characterised in the susceptibility to CD (Barrett *et al.*, 2008, Franke *et al.*, 2010). Interestingly, the *IL-10* gene has been identified as a key susceptibility gene, with IL-10-knockout mice providing one of the best models for the study of IBD (Franke *et al.*, 2010).

There is some evidence to suggest that the regulation and/or mutation of genes encoding pattern recognition receptors (PRRs) could potentially play a major role in IBD. Two independent, yet simultaneous, studies undertaken by Hugot *et al.* (2001) and Ogura *et al.*, (2001) both found that mutations of the *NOD-2* gene product confers significant susceptibility to CD. The mutants exhibited altered microbial antigen recognition through the NOD-2 receptor and/or over-activation of NF- κ B in monocytes (Hugot *et al.*, 2001). NOD-2 has also been shown to be significantly up-regulated in young CD patients (Berrebi *et al.*, 2003). Additionally, the LPS receptor TLR-4 is typically scarcely detectable in intestinal tissue, however, its expression is greatly up-regulated in IBD-inflamed tissues (Cario and Podolsky, 2000). Furthermore, polymorphism mutations in the *TLR-4* gene have been found in both CD and UC patients, and could potentially predispose individuals to IBD (Franchimont *et al.*, 2004). Rakoff-Nahoum *et al.* (2006) have also shown that the adaptor molecule, MyD88, which is responsible for intracellularly transmitting TLR stimulation, could be a major contributing factor to IBD, as MyD88-deficiency results in colitis-free animals in inflammatory models, such as *IL-10*^{-/-} mice.

The loss of microfloral tolerance by the intestinal immunity was originally demonstrated by Duchmann *et al.* (1995), who challenged LPMCs (lamina propria mononuclear cells) from non-inflamed and IBD-inflamed intestinal tissues, with both heterologous and autologous microfloral sonicates. They found that immune cells from non-inflamed intestinal tissue were reactive to non-indigenous microfloral sonicates, but were unresponsive to indigenous sonicates (ie. self-tolerance), whereas the equivalent cells isolated from an IBD-inflamed intestine were hyperresponsive to both sonicate samples (Duchmann *et al.*, 1995). Further to this, ~50 % of CD patients show positive serological responses to enteric-origin antigens, for example, outer membrane proteins (OMPs) (Landers *et al.*, 2002)

and flagellin (Lodes *et al.*, 2004; Targan *et al.*, 2005), which the host immune system would normally tolerate (Sartor, 2008).

One theory for the loss of host immune tolerance is the dysbiosis of the commensal microflora. A pioneering metagenomic study by Frank *et al.* (2007) showed decreased levels of *Firmicutes* and *Bacteroidetes* in the microflora of IBD patients, suggesting a fundamental compositional change in the microflora. Additionally, a study by Rath *et al.* (2001) showed that some groups of enteric microflora have increased IBD-inducibility when compared to others. Another hypothesis suggests that microbial pathogens could elicit the induction of IBD, with an increase in adherent and invasive *E. coli* (AIEC) seen in IBD patients (Darfeuille-Michaud *et al.*, 1998). In addition, *Mycobacterium avium* sp. *paratuberculosis* (MAP), the aetiological agent in spontaneous granulomatous enterocolitis in ruminants such as cattle and sheep (Harris and Barletta, 2001), presented a strong candidate, as it was isolated from inflamed tissue samples of CD patients (Chiodini *et al.*, 1984, Autschbach *et al.*, 2007). However, a long treatment course of antimycobacterial antibiotics did little to alleviate the symptoms in CD patients (Selby *et al.*, 2007), thus dispelling the theory of MAP as a causative agent.

In contrast to this, there is ever-increasing evidence of innate defence abnormalities which result in susceptibility to IBD, via increased mucosal association of bacteria (Swidsinski *et al.*, 2002). CD patients exhibit diminished physico-chemical barriers with a reduced colonic mucus layer (Cobrin and Abreu, 2005) and dramatically reduced expression of defensins HD-5 and HD-6 in the ileum (Wehkamp *et al.*, 2005). This decreased antimicrobial activity results in loss of regulation of commensal microflora and increased epithelial exposure (Wehkamp *et al.*, 2005). Moreover, the ‘leaky gut’ hypothesis proposes that loss of epithelial integrity predisposes to IBD (Schmitz *et al.*, 1999; Soderholm *et al.*,

2002); however, a later study by Collett *et al.* (2008) suggested that a ‘leaky’ epithelium develops only when the disease is established.

Mucosal adaptive immunity also demonstrates malfunctions in IBD, with T-cells resistant to apoptosis found in CD, thus resulting in an increased and prolonged inflammatory response (Cobrin and Abreu, 2005). T-cell stimulation is also enhanced due to increased numbers of intraepithelial DCs which produce higher levels of proinflammatory cytokines, such as IL-12 and IFN γ , and possess increased class II MHC antigen in IBD-inflamed intestinal tissues (Bene *et al.*, 2011).

Environmental factors have also been implicated in the pathophysiology of IBD, but with distinctive variability between the two major forms of IBD. For example, smoking has been suggested to double the likelihood of developing CD (Loftus, 2004), but, conversely, is associated with exacerbation of UC (Baumgart and Carding, 2007). Similarly, there is a 69 % decrease in risk of developing UC subsequent to undergoing an appendectomy (Loftus, 2004), but significantly increased risk of stricture development in CD (Baumgart and Carding, 2007).

Regardless of predispositions and pathogeneses, IBD culminates in the increased migration and activation of monocytes, macrophages, granulocytes and lymphocytes from the blood stream to the mucosa, leading to continued inflammation and perpetuation of disease (Banks *et al.*, 2003; Atreya and Neurath, 2010).

1.5 Bacteria and their extracellular products

1.5.1 Bacterial cell walls

Bacteria can be separated into two distinct categories: Gram-positive and Gram-negative bacteria (Madigan and Martinko, 2006). The original distinction between the two comes from the staining technique serendipitously discovered in 1884 by the Danish bacteriologist,

Christian Gram (Gram, 1884). Gram staining utilises two staining steps with crystal violet and saffranin, respectively, and an intermediary wash step with alcohol. The basis of the differentiation between two types of bacteria relies upon the chemical composition and structural configuration of their cell walls (Salton, 1963). Gram-negative bacteria possess a lipid-rich outer membrane (OM) and a thin peptidoglycan layer and, as a result, the primary stain, crystal violet, is washed from the cells with the decolourising alcohol wash, leaving the secondary stain, saffranin, to colour the cells pink (Beveridge, 1999). In contrast, the thick peptidoglycan layer of Gram-positive bacteria retains the crystal violet stain, colouring the cells purple (Beveridge, 1999). Figure 1.3 highlights the difference in the two types of bacterial cell wall.

The composition of the Gram-positive cell wall is relatively simple and consists mainly of the cytoplasmic phospholipid membrane and the characteristically thick layer of peptidoglycan (PGN) (Figure 1.3a), which accounts for 30-70 % of the mass of the cell wall (Schleifer and Kandler, 1972). However, a number of accessory molecules, such as surface proteins, teichoic and lipoteichoic acids and carbohydrates, also reside within the PGN layer (Navarre and Schneewind, 1999).

Gram-negative cell walls are much more complex than their Gram-positive equivalents, as they constitute a multilayer structure consisting of two membrane bilayers which sandwich a concentrated gel-like matrix (the periplasm) and a layer of peptidoglycan (Beveridge, 1999) (Figure 1.3b). Compared to the Gram-positive cell wall, the peptidoglycan layer is thin and only accounts for <10 % of the whole cell wall (Schleifer and Kandler, 1972). The intermediary periplasmic layer contains a diverse mix of periplasmic enzymes, trafficking proteins and outer membrane- or peptidoglycan layer-directed proteins (Beveridge, 1999), all of which contribute to its gel-like consistency. The inner of the two membrane bilayers, the cytoplasmic membrane (CM), is made up solely of phospholipids and

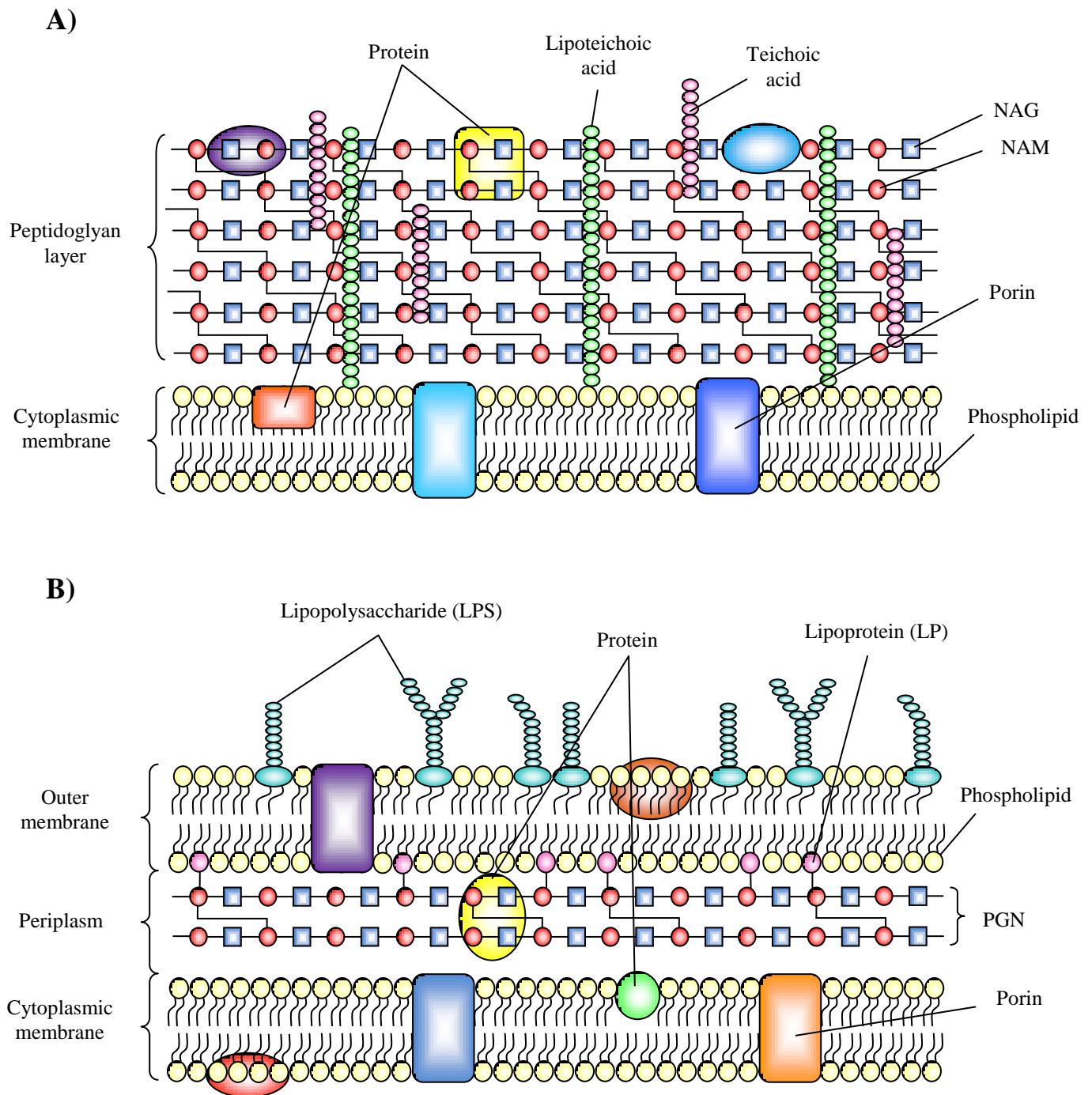


Figure 1.3 – Cross-sectional structures of bacterial cell walls. This illustrates the characteristic thick peptidoglycan layer, and its associated molecules, of the Gram-positive cell wall (**A**). On the other hand, the Gram negative cell wall exhibits its two membrane bilayers with the periplasm and thin peptidoglycan layer between them (**B**). PGN = peptidoglycan, NAG = N-acetylglutamic acid, NAM = N- acetylmuramic acid.

proteins and acts much like the cytoplasmic membrane of Gram-positive bacteria. However, the outer bilayer, the outer membrane (OM), is composed of mainly of lipopolysaccharides, phospholipids and small amounts of proteins and lipoproteins, and, thus, behaves very differently from typical phospholipid bilayer membranes (Beveridge, 1999). Lipopolysaccharide (LPS) molecules are negatively charged and require the sequestration of magnesium ions (Mg^{2+}) to decrease electrostatic repulsions between them, thus maintaining tight lateral interactions and, ultimately, OM integrity (Bishop, 2005). LPS molecules also contain saturated acyl chains which decrease the fluidity of the membrane (Bishop, 2005). Tight lateral bonds and low fluidity result in a membrane permeable only to lipophilic solutes and detergents, however, transmembrane porins allow the selective bidirectional transportation of other molecules (Bishop, 2005). The asymmetric distribution of the OM components results in the presentation of highly antigenic LPS molecules on the bacterium's external surface, whereas the majority of phospholipids are found on the periplasmic face (Beveridge, 1999). The lipoproteins are also located on the periplasmic surface and function to bind the thin peptidoglycan layer to the OM. Some of the major antigenic differences between Gram-positive and Gram-negative cell walls are discussed below.

1.5.2 Endotoxins (lipopolysaccharides)

The German bacteriologist, Richard Pfeiffer, coined the term 'endotoxin' in 1892 when he demonstrated that heat-killed bacteria of the cholera-inducing *Vibrio cholera* caused toxic shock in guinea pigs (Pfeiffer, 1892; Alexander and Rietschel, 2001). He subsequently theorised that the heat-stable toxin was present inside the bacterial cell and consequently named it 'endotoxin' to distinguish it from the already documented exotoxins of *V. cholera* (Alexander and Rietschel, 2001). It has since been discovered that endotoxic activity of

Gram-negative bacteria originates from lipopolysaccharides (LPS) which constitute the majority (~75 %) of their outer membrane (Rietschel *et al.*, 1994). LPS is an amphiphilic membrane phospholipid (Fenton and Golenbeck, 1998) which is essential for cell viability and outer membrane permeability (Rietschel *et al.*, 1994). It also plays a key role in protection of the bacterium against host immune defences, enzymatic degradation and antibiotic attack (Holst *et al.*, 1996). Since only the *Sphingomonas* genus is found to lack LPS (Alexander and Rietschel, 2001), its ubiquitous expression in other Gram-negative bacteria presents the mammalian innate immune system with a major target (Erridge *et al.*, 2002). However, LPS is not actively secreted by Gram-negative bacteria, and is normally only released in small amounts during mitotic division (Caroff and Karibian, 2003).

1.5.2.1 General structure of LPS

LPS is a glycolipid macromolecule consisting of three domains; the distal hydrophobic O-specific chains, or O-antigens, which extend into the aqueous media; the interconnecting core region; and the hydrophobic lipid A region which acts as the membrane anchor (Bishop, 2005) (Figure 1.4).

The O-specific chain regions of LPSs are highly diverse polymers of repeating saccharide subunits which vary greatly in different bacterial strains (Erridge *et al.*, 2002). They are not essential for the viability of the bacterium, as not all Gram-negative LPSs possess O-antigens (Bishop, 2005). O-antigen-lacking strains of bacteria are termed rough (R-) serotypes due to their characteristic colony morphology which is distinct from the morphology of smooth (S-) serotype colonies (Alexander and Rietschel, 2001). Despite their non-essential nature, O-antigens have demonstrated a highly beneficial role in bacterial survival during infection of the host, aiding in the avoidance of phagocytosis by macrophages (Rietschel *et al.*, 1994) and circumvention of the lytic action of the host complement system

and antibiotics (Caroff and Karibian, 2003). However, paradoxically, O-antigens present a major target for the host's antibody response, as they represent the extreme outer limits of the bacterial cell (Erridge *et al.*, 2002).

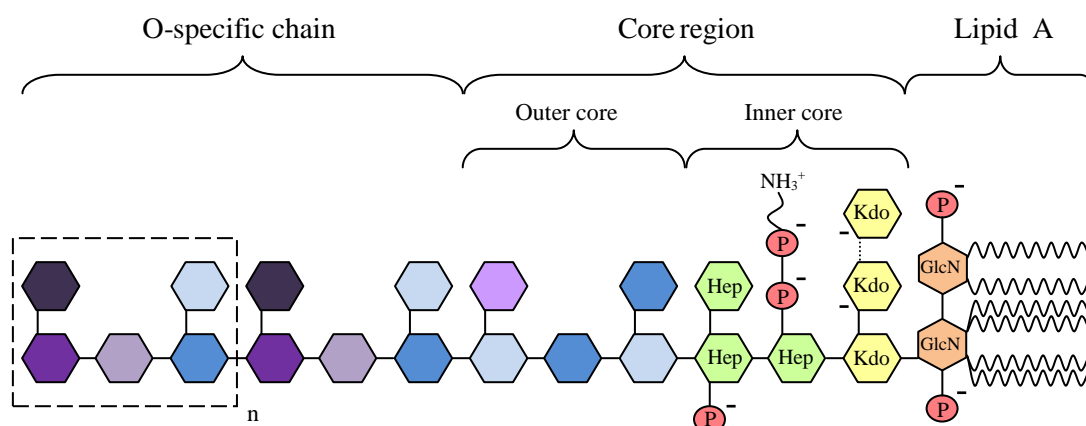


Figure 1.4 – General structure of LPS from Gram-negative enterobacteria. GlcN = glucosamine, Kdo = 2-keto-3-deoxy-D-manno-octonate, Hep = D-glycero-D-manno-heptose. Adapted from Alexander and Rietschel, (2001).

The core region is separated into two distinct domains, the inner and outer core regions (Holst *et al.*, 1996). The inner core is very well conserved within a genus or family of Gram negative bacteria (Raetz and Whitfield, 2002) and is generally composed of LPS-specific D-glycero-D-manno-heptose (Hep) and 2-keto-3-deoxy-D-manno-octonate (Kdo) (Rietschel *et al.*, 1994). This inner core domain is essential for bacterial viability, with the minimum structure consisting of one Kdo residue (Rietschel *et al.*, 1994). The outer core has a more diverse structure and typically consists of combinations of hexose sugars, such as glucose and galactose (Erridge *et al.*, 2002). Both core sugars are often substituted with negatively charged groups such as phosphates and pyrophosphates, giving the LPS molecule an overall negative charge (Erridge *et al.*, 2002).

The glycolipid membrane anchor, lipid A, represents the biologically active moiety of LPS, as both free and synthetic lipid A molecules reproduce the effects of whole LPS

(Galanos *et al.*, 1985). The lipid A domain consists of a phosphorylated β -1,6-linked glucosamine (GlcN) disaccharide (Netea *et al.*, 2002), to which up to 4 acyl chains can be attached. Furthermore, the acyl chains can be substituted with fatty acids, giving a maximum of 7 acyl chains in the lipid A region (Erridge *et al.*, 2002). The number and length of the constituent acyl chains determines the three-dimensional shape of lipid A, and is linked to the overall endotoxic potential of the LPS molecule (Schromm *et al.*, 2000). High biological activity has been linked to non-cylindrical lipid A molecules (due to their favourable steric interactions with the LPS receptor), whereas cylindrical-shaped lipid A molecules have low or no endotoxic activity (Schromm *et al.*, 2000).

1.5.2.2 Immunostimulation by LPS

In a healthy individual, the basal systemic concentration of LPS in the human body can be in the range of 3-10 pg/ml (Alexander and Rietschel, 2001). Accordingly, the highly evolved innate immune system can detect and, indeed, degrade these concentrations of LPS (Hoffman and Natanson, 1997; Ulevitch and Tobias, 1999) in a phenomenon known as 'LPS tolerance'. LPS tolerance has been shown to aid in the defence against subsequent bacterial invasion by the parent strain (Hoffman and Natanson, 1997). However, larger quantities of LPS, often released by bacterial lysis during infection (Caroff and Karibian, 2003), can have a highly detrimental effect on the host, resulting in fever, increased heart rate, septic shock and, ultimately, death from multiple organ failure and systemic inflammatory response (Hoffman and Natanson, 1997; Caroff and Karibian, 2003). LPSs do *not* elicit their toxic effect by the killing of host cells, or even by the inhibition of host cellular function, but via the active inflammatory responses of host cells (Rietschel *et al.*, 1994).

The first stage in host recognition of LPS is the binding of the acute phase reactant, LPS-binding protein (LBP) (Hailman *et al.*, 1994), which predominantly originates from the

liver and freely circulates in the blood (Fenton and Golenbeck, 1998). The main function of LBP is to opsonise and deliver LPS to the cluster of differentiation (CD)14 receptor, with each LBP molecule chaperoning 10 LPS molecules to the receptor (Hailman *et al.*, 1994). CD14 is a member of the toll-like receptor (TLR) family (Triantafilou and Triantafilou, 2002) and exists in two forms, membrane-bound (mCD14) and soluble (sCD14), both of which are able to mediate the activation of host cells (Pugin *et al.*, 1993). Guha and Mackman (2001) suggested that mCD14 is important for LPS recognition, as CD14-deficient mice showed LPS hyposensitivity, whereas overexpression resulted in LPS hypersensitivity and increased susceptibility to endotoxic shock. However, despite being embedded within the cell membrane, mCD14 does not possess a cytoplasmic domain, and therefore CD14 lacks the ability to activate a transmembrane activation signal (Triantafilou and Triantafilou, 2002). This implies that another receptor confers responsiveness to LPS. Poltorak *et al.* (1998) suggested that Toll-like receptor (TLR)-4 was responsible for LPS sensitivity, as they found the human *TLR-4* gene was homologous to the murine *Lps* gene, which was shown to control leukocyte response to LPS (Linder *et al.*, 1988). Subsequently, Hoshino *et al.* (1999) demonstrated TLR-4 to be the translational product of the *Lps* gene and generated TLR-4-deficient mice which, consequently, lacked responsiveness to LPS. There was some speculation that TLR-2 could also play a role in LPS responsiveness (Kirschning *et al.*, 1998, Yang *et al.*, 1998), however, this was soon nullified when meticulous repurification of LPS, removing any lipoprotein contaminants, showed that TLR-4 was responsible (Hirschfield *et al.*, 2000). However, TLR-4 does not work alone in LPS recognition, a co-factor, MD-2, forms a receptor complex, which induces an intracellular signal transduction cascade once the CD14-bound LPS is transmitted (Shimazu *et al.*, 1999; Heumann and Roger, 2002).

The innate immune response to LPS is orchestrated by CD14-expressing immune cells such as monocytes and macrophages, which react to the presence of LPS by producing

proinflammatory cytokines such as TNF- α , IL-6 and IL-8 (Guha and Mackman, 2001). However, CD14-deficient cells, such as endothelial cells, are also able to respond to LPS in the presence of serum (Hailman *et al.*, 1994). Intestinal epithelial cell lines have also revealed sensitivity to LPS with the stimulation of the proinflammatory cytokine, IL-8, in both HT29 cells (Schuerer-Maly *et al.*, 1994; Smirnova *et al.*, 2003) and Caco-2 cells (Huang *et al.*, 2003). Conversely, Berg *et al.* (1995) demonstrated that, in murine models, LPS was also able to induce an increase in serum concentrations of the anti-inflammatory cytokine, IL-10, thus suggesting a role in LPS tolerance. However, the same study showed that LPS tolerance was not dependent on IL-10, but the process was potentiated by the cytokine (Berg *et al.*, 1995).

1.5.3 Membrane vesicles (MVs)

Membrane vesicles (MVs) are small (50-250 nm diameter), spherical, bilayered membranous structures (Beveridge, 1999) produced by Gram-negative bacteria. MVs have been found to be produced by every Gram-negative bacteria investigated to date (Ellis and Kuehn, 2010) and are consequently found in a diverse range of environments, from liquid and solid lab cultures to river beds and waste water pipes (Schooling and Beveridge, 2006). MVs have also been isolated *in vivo*, at both the site of bacterial infection (Fiocca *et al.*, 1999; Keenan *et al.*, 2000) and in distant organs of infected hosts (Dorward *et al.*, 1991).

MVs are unique to Gram-negative bacteria as they originate from the outer membrane (OM), a feature absent from the cell wall of Gram-positive bacteria (see Section 1.5.1). The composition, conformation and surface chemistry of MVs represent the intact outer membrane of Gram-negative bacteria, but on a smaller scale (Beveridge, 1999, Schooling and Beveridge, 2006). Lipopolysaccharides (LPSs), outer membrane proteins (OMPs), phospholipids and periplasmic proteins all constitute MVs (Beveridge, 1999; Kesty and

Kuehn, 2004) and proteins such as transmembrane porins, murein hydrolases, transporter proteins, virulence factors and motility-related proteins have all been identified in MVs by proteomic studies (Lee *et al.*, 2008).

1.5.3.1 Biogenesis of MVs

The biogenesis of MVs is, as yet, unknown (Kulp and Kuehn, 2010), however they are not an artefact of cell division or lysis, nor simply a consequence of membrane instability (McBroom *et al.*, 2006). Currently, there are three main hypotheses for the mechanism of MV formation. The first was influenced by a study undertaken by Wensink and Witholt, which demonstrated that MVs isolated from *Escherichia coli* contained little lipoprotein content and a lipoprotein-deficient mutant of *E. coli* showed excessive MV production (Wensink and Witholt, 1981). Consequently, they theorised that MVs are derived from areas of the Gram-negative outer membrane which have few lipoprotein links with the underlying peptidoglycan layer (Wensink and Witholt, 1981). However, this hypothesis implies a non-homogeneous distribution of lipoprotein in the OM, which is, as Wensink and Witholt themselves stated, highly unlikely, as the OM contains a very high density of lipoprotein (50,000-100,000 molecules of lipoprotein/ μm^2 (Osborn and Wu, 1980)). Subsequently, they postulated a more likely scenario, in which the OM expands faster than the underlying peptidoglycan layer, resulting in membrane blebbing and subsequent pinching off of the OM, thus forming a membrane vesicle (Wensink and Witholt, 1981). The second hypothesis of MV biogenesis proposed that turgor pressure from the periplasm results in outer membrane blebbing and MV formation (Zhou *et al.*, 1998). Zhou *et al.* stated that, during the mitotic division of Gram-negative bacteria, the turnover of the peptidoglycan layer (underlying the OM) produces low molecular mass muramyl peptides which cannot penetrate the OM and so accumulate within the periplasmic space (Zhou *et al.*, 1998). Furthermore, they hypothesised

that the accumulation of muramyl peptides exerts turgor pressure, herniating the OM and producing membrane blebs which subsequently pinch off forming MVs. However, their theory was based solely on the discovery of peptidoglycan-originating muramic acid in MVs of *P. gingivalis*. The third hypothesis of MV biogenesis proposes that a system analogous to budding mechanisms observed in eukarya and archaea is involved in MV formation (Kulp and Kuehn, 2010). This theory relies on curvature-inducing molecules aggregating and causing bulging of the OM, a process which would result in the enrichment of these molecules within the MVs. This model was upheld by the discovery that the outer leaf of the bilayer membrane of *Pseudomonas aeruginosa* MVs mainly consisted of the negative B-band LPS molecule, as opposed to the more neutral A-band LPS (Kadurugamuwa and Beveridge, 1995). It was subsequently suggested that the electronegative charges between the B-band LPS molecules caused charge-to-charge repulsion, resulting in OM instability, blebbing and MV formation (Mashburn-Warren and Whitely, 2006). Currently, all three hypotheses are accepted as possible mechanisms and, despite their divergence, all three agree on the latter stages, i.e. the budding and pinching off of the OM to release the MVs (Figure 1.5).

1.5.3.2 Biological roles of MVs

A number of functions have been ascribed to MVs, with roles in fundamental cell processes such as periplasmic equilibrium maintenance and protein transport suggested (McBroom and Kuehn, 2007). However, a direct accountability in the virulence of Gram-negative bacteria is the most strongly supported proposal (Ellis and Kuehn, 2010), thus, the best characterised MVs are those originating from pathogens (Kuehn and Kesty, 2005) (see Section 1.5.3.3).

However, in addition to virulence, Gram-negative bacteria could utilise MVs in a protective role, as Ciofu *et al.* (2000) found that the antibiotic-denaturing enzyme β -lactamase was preferentially packaged inside the MVs of *P. aeruginosa*. The same MVs were

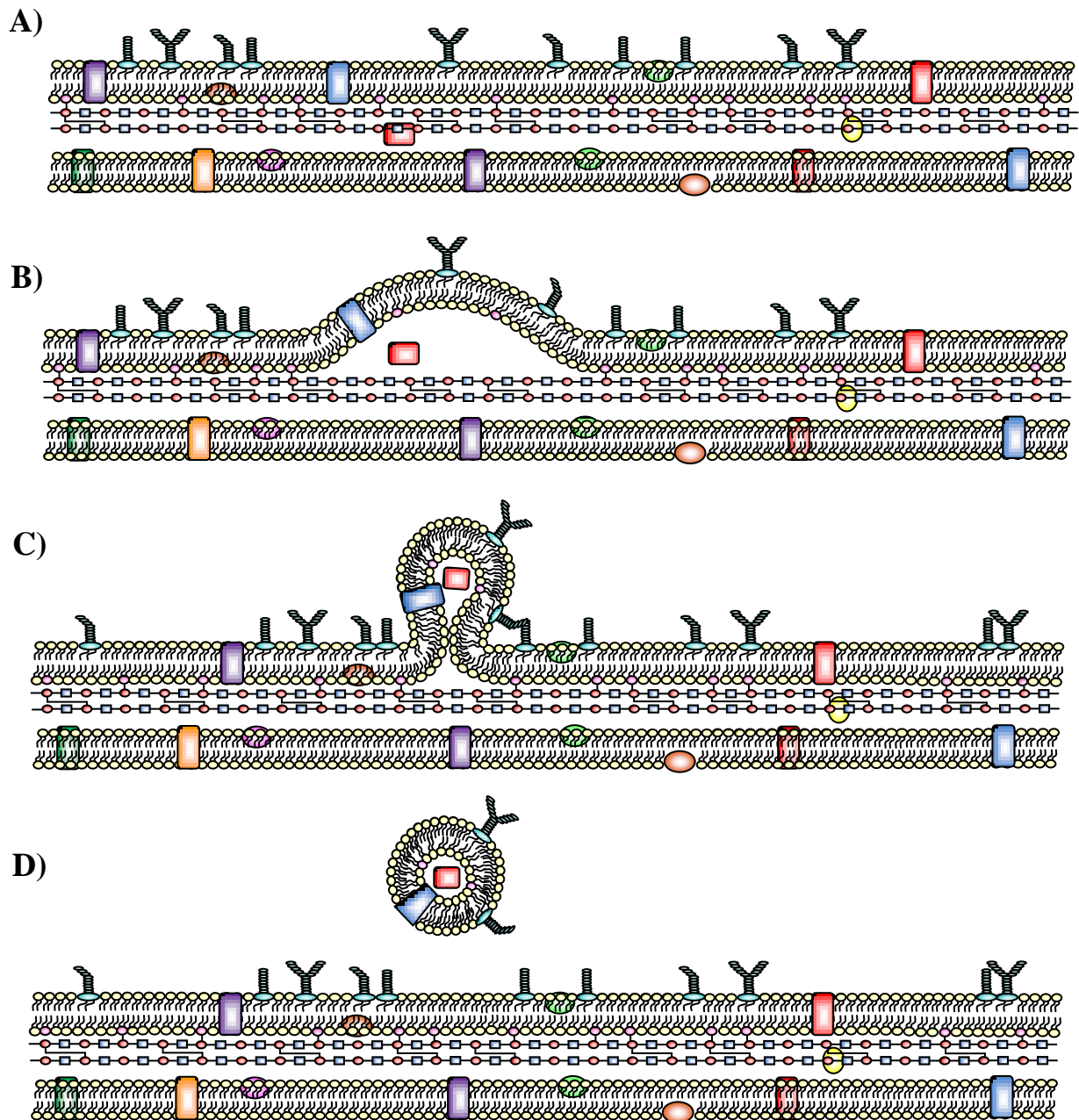


Figure 1.5 – Formation of membrane vesicles. The specific mechanism of MV biogenesis is still unknown; however the general process is summarised above. The outer membrane (OM) of the Gram-negative cell wall (A) undergoes an unknown initiation process which results in the bulging out of the OM (B). The protuberance is subsequently pinched off (C) and the vesicle is released from the OM, which returns to its ‘normal’ state (D).

also seen to contain outer membrane porins which allowed the passage of β -lactam antibiotic molecules into the enzyme-containing luminal cavities of the MVs, where they were subsequently digested within the MV lumen, thus greatly reducing their bactericidal activity. Additionally, Manning and Kuehn (2011) proposed that MVs represent a defence utilised by Gram-negative bacteria against outer membrane-acting bacterial antimicrobial peptides (AMPs), such as polymyxin B and colistin. They documented an up-regulation of MV production, in *E. coli*, in response to polymyxin B and colistin which subsequently neutralised the further effectiveness of the AMPs as they adsorbed to the MVs rather than the bacteria themselves. A similar effect was seen in T4 bacteriophage-challenged *E. coli*, with the infectivity of the phage reduced by ~90 % as it irreversibly bound to MVs.

Mashburn-Warren and Whitely (2006) have suggested that MVs could also represent a cell- to-cell communication mechanism analogous to the vesicle trafficking system utilised by multicellular eukaryotic organisms. Their report showed that the extracellular signalling molecule, *Pseudomonas* quinolone signal (PQS), which induces the transcription of various virulence genes in *P. aeruginosa*, mediates its own packaging into MVs. The MVs then deliver the quorum sensing molecule from the parent bacterium to other bacteria in the surrounding area, inducing the production of virulence factors such as pyocyanin (Mashburn-Warren and Whitely, 2006).

Additionally, MVs could represent a novel mechanism of horizontal gene transfer between bacteria. Both plasmid and chromosomal DNAs have been found to subsist in MVs isolated from *Neisseria gonorrhoeae* (Dorward *et al.*, 1989), *E. coli* (Yaron *et al.*, 2000) and *P. aeruginosa* (Renelli *et al.*, 2004). Additionally, Dorward *et al.* (1989) demonstrated the transformation, via MVs, of antibiotic resistance genes in strains of *N. gonorrhoeae* and this was followed by Yaron *et al.* establishing the transmission of virulence factor genes, from pathogenic to non-pathogenic strains of *E. coli*, through MVs (Yaron *et al.*, 2000). However,

P. aeruginosa MVs did not transfer antibiotic resistance genes between strains of the pathogen, despite attaching and subsequently fusing with the OM (Renelli *et al.*, 2004).

MVs are also very likely to function as a bridging factor within sessile bacterial communities, called biofilms (Costerton *et al.*, 1999), with their presence found in both lab-grown *P. aeruginosa* monoculture biofilms and in naturally occurring mixed species biofilms, in which Gram-negative bacteria were present (Schooling and Beveridge, 2006). Interestingly, MVs were not only maintained in substantial numbers within the matrix, but were also actively released from the biofilm into the external milieu.

1.5.3.3 Virulence of MVs

As mentioned previously, only MVs isolated from pathogenic bacteria have been studied; in particular, MVs from the respiratory pathogen *P. aeruginosa* (Kadurugamuwa and Beveridge, 1995; 1996; 1997; Li *et al.*, 1998; Bauman and Kuehn, 2006; 2009; Ellis *et al.*, 2010). In early studies of MVs, Kadurugamuwa and Beveridge proposed a predatory role for *P. aeruginosa* PAO1 MVs against other bacteria (Kadurugamuwa and Beveridge, 1996). Peptidoglycan hydrolase enzymes, found within the MVs, were shown to be delivered to the periplasm of other strains of *P. aeruginosa*, where they digested the thin peptidoglycan layer and catalysed cell lysis. Li *et al.* further confirmed this, showing *P. aeruginosa* PAO1 MVs to have lytic activity against a range of other Gram-negative and Gram-positive bacteria (Li *et al.*, 1998). The same study also found that MVs from numerous other Gram-negative bacteria, such as *Bacillus subtilis*, *Shigella flexneri* and *Proteus vulgaris*, all showed similar lytic abilities over the same range of recipient bacteria (Li *et al.*, 1998). Therefore, the production of MVs would be a very useful tool for the successful establishment of a bacterial species within an environmental niche (Unal *et al.*, 2011) as the lysis of other strains of

bacteria would both remove the competition and liberate a rich source of nutrients (Li *et al.*, 1998).

Previous to the recognition of MVs as a means to aid bacterial colonisation, Kadurugamuwa and Beveridge had first suggested the virulent nature of MVs due to the enrichment of antigenic LPS molecules and the inclusion of host tissue-destructive enzymes, such as elastases, in MVs isolated from *P. aeruginosa* (Kadurugamuwa and Beveridge, 1995). The findings of Kesty *et al.* (2004) further supported this when they showed that enterotoxigenic *E. coli* (ETEC) MVs preferentially packaged heat-labile (LT) toxin in their luminal space, protecting it from extracellular enzymatic activity, and later delivering it directly to the cytoplasm of target cells. A similar system was also seen in *Helicobacter pylori*, with its MVs encapsulating and transporting its major virulence factor, *H. pylori* vacuolating toxin (Parker *et al.*, 2010). The immunomodulatory potential of MVs was recognised when Ismail *et al.* (2003) showed MVs isolated from *H. pylori* elicited IL-8 release in human gastric epithelial cells. The immunostimulatory potential of *P. aeruginosa* MVs was uncovered shortly afterwards as it was demonstrated that MVs from the respiratory pathogen stimulated IL-8 in human lung epithelial cells (Bauman and Kuehn, 2006), following their attachment and internalisation (by endocytosis) (Bauman and Kuehn, 2009). Ellis *et al.* (2010) also showed *P. aeruginosa* MVs to be potent stimulators of pro-inflammatory responses in murine macrophages. More recently, MVs isolated from *H. pylori* have been shown to elicit IL-8 responses in human gastric epithelial cell lines through the novel delivery of peptidoglycan to the intracellular MAMP receptor, NOD1 (Kaparakis *et al.*, 2010).

Despite the high load of Gram-negative bacteria in the lumen, intestinal commensal strains have (to the author's knowledge) yet to be considered in the study of MVs. Also, the interaction of MVs with intestinal epithelial cells have, surprisingly, been little studied, with

only Kesty *et al.* (2004) utilising them to demonstrate the toxin-delivering activity of MVs isolated from ETEC.

1.5.4 Peptidoglycan

Peptidoglycan (PGN) is an essential cell wall component in virtually all bacteria and is especially abundant in Gram-positive bacteria, accounting for 30-70 % of the cell wall mass (Schleifer and Kandler, 1972). It is a mesh-like polymer consisting of $\beta(1-4)$ -linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), crosslinked by short peptides and is responsible for the maintenance of cell morphology and resistance to osmotic forces (Dziarski, 2003). As a consequence of its ubiquity in bacteria, its substantial abundance in Gram-positive bacteria and its absence from eukaryotic cells, PGN presents the perfect target for the host innate immune system (Dziarski, 2003). Initially, it was hypothesised that TLR-2 mediated cellular sensitivity to PGN in macrophages (Schwandner *et al.*, 1999; Takeuchi *et al.*, 1999; Wang *et al.*, 2001), and that responsiveness was enhanced by the co-receptor CD14 (Schwandner *et al.*, 1999; Iwaki *et al.*, 2002). Iwaki *et al.* (2002) also generated a soluble form of TLR-2 and demonstrated that PGN purified from *Staphylococcus aureus* directly bound to the receptor. However, an infamous study by Trovassos *et al.* (2004) challenged the proposed stimulation pathway, claiming TLR-4, not TLR-2 conferred cellular responsiveness to purified PGN. Nevertheless, the re-evaluation of the phenomenon that followed conclusively demonstrated that TLR-2 was essential for the stimulation of macrophages by PGN and suggested the results observed by Trovassos *et al.* (2004) were due to the destructive nature and non-completion of their purification methods (Dziarski and Gupta, 2005).

Due to its high release during infection (Dziarski and Gupta, 2005), and potent immunological activity (NF- κ B activation and subsequent IL-8 release) in mouse and human

macrophages (Schwandner *et al.*, 1999; Takeuchi *et al.*, 1999; Wang *et al.*, 2001), PGN is potentially a major virulence factor in Gram-positive bacterial infections. Melmed *et al.* (2003) observed that both colonic epithelial tissue and colonic epithelial cells lines (HT29, Caco-2 and T84 cells) constitutively expressed TLR-2 mRNA, suggesting a role in PGN detection in the intestine (Melmed *et al.*, 2003). However, the epithelial cells lines were all largely unresponsive to TLR-2 ligands (such as PGN and lipoteichoic acid (LTA) (Section 1.5.11)) and only TLR-2-transfected Caco-2 cells exhibited activation by TLR-2 agonists (Melmed *et al.*, 2003). Furthermore, Furrie *et al.* (2005) showed TLR-2 mRNA expression in colonic epithelial tissue was restricted to crypt cells. However, they also showed that, in contrast to the findings of Melmed *et al.* (2003), TLR-2 mRNA was up-regulated in human colonic epithelial cell lines, HT29 and Caco-2 cells, in response to co-culture with Gram-positive bacteria (Furrie *et al.*, 2005).

1.5.5 Lipoprotein

Lipoproteins (LPs) are proteins which contain lipid moieties covalently bound to an N-terminal cysteine residue (Braun and Wu, 1994). LPs are a key component in the cell wall of Gram-positive bacteria (Sutcliffe and Russell, 1995) and the outer membrane of Gram-negative bacteria, particularly in bacteria of the *Enterbacteriaceae* family, such as *E. coli* (Zhang *et al.*, 1998). Gram-negative bacteria naturally secrete LPs, from their outer membrane, into the surrounding media and lysis further increases the release (Zhang *et al.*, 1998). Brightbill *et al.* (1999) elucidated that cellular responsiveness to bacterial lipoproteins in human macrophages is imparted by Toll-like receptor (TLR)-2. This was subsequently confirmed by Wang *et al.* (2002), who demonstrated that pre-treatment of human monocytes with low concentrations of LP imparts TLR-2 tolerance that protects against subsequent treatment with higher concentrations of LP. However, it was later discovered that TLR-2

actually forms a heterodimer with TLR-1 to confer cell responsiveness to bacterial lipoproteins in murine macrophages (Takeuchi *et al.*, 2002).

LPs have been shown to elicit proinflammatory cytokine release in mouse and human macrophages (Zhang *et al.*, 1998) and human whole blood (Karched *et al.*, 2008), and can even activate human neutrophils (Soler-Rodriguez *et al.*, 2000). Additionally, spirochetal LPs, from *Treponema pallidum* and *Borrelia burgdorferi*, have been implicated in the pathogenesis of syphilis and Lyme disease, respectively (Sellati *et al.*, 1998). Consequently, bacterial LPs clearly present a significant antigenic threat, however, few, if any, studies have been undertaken to investigate lipoproteins with intestinal epithelial cells.

1.5.6 Lipoteichoic acid

Lipoteichoic acid (LTA) is a membrane-associated, amphiphilic polymer which extends from the cytoplasmic membrane, through the cell wall and to the outer surface of Gram-positive bacteria (Buckley *et al.*, 2006). During normal growth, LTA regulates a number of autolytic enzymes, which allow the mitotic division of Gram-positive bacteria (Ginsburg, 2002), and has also been demonstrated to aid bacterial adhesion to intestinal epithelial cells (Granato *et al.*, 1999). Naturally, LTA is only released in small amounts (Ginsburg, 2002); however cell lysis will inevitably increase its liberation into the surrounding media. Pathogen-derived LTA is well known to be immunologically active and has previously been demonstrated to stimulate pro-inflammatory cytokines, such as IL-8, from peripheral blood mononuclear cells (PBMCs), such as macrophages (Standiford *et al.*, 1994). Additionally, LTA from potentially probiotic strains of *Lactobacillus* have also been shown to elicit a proinflammatory cytokine response in PBMCs (Vidal *et al.*, 2002). Despite this, Vidal *et al.* (2002) also showed that the same lactobacilli LTAs were unable to stimulate a proinflammatory response in the intestinal

epithelial cell line, HT29 cells, and actively inhibited *E. coli*- and LPS-induced IL-8 release in these cells.

There is some debate as to which of the Toll-like receptors (TLRs) confers host cellular responsiveness to LTA, as two very highly cited articles tell contrasting stories. Schwandner *et al.* (1999) demonstrated that human embryonic kidney cells were activated via TLR-2, however, Takeuchi *et al.* (1999) disputed this, suggesting that TLR-4 was responsible as they showed that TLR-2-deficient mice were still responsive to LTA, whereas TLR-4-deficient mice were not.

1.5.7 CpG-DNA

Bacterial DNA contains a ~20-fold greater frequency of unmethylated 2'-deoxyribo(cytidine-phosphate-guanine) (CpG) dinucleotides than vertebrate DNA (Ewaschuk *et al.*, 2007), predisposing it to microbial-associated molecular pattern (MAMP) activity in mammalian immune cells (Bauer *et al.*, 2001). Methylated bacterial DNA loses its stimulatory potential (Ewaschuk *et al.*, 2007), thus confirming that its MAMP activity is attributable to a greater expression of unmethylated CpG motifs. In addition, the stimulatory effects of bacterial DNA, on mammalian immune cells, can be mimicked by CpG-containing synthetic oligodeoxynucleotides (CpG-ODNs) (Dalpke *et al.*, 2006). Dalpke *et al.* (2006) also showed that the immunomodulatory potential of bacterial DNA is genera specific and dependent on the CpG content of the bacterial genome. For example, DNA from the pathogenic *Pseudomonas aeruginosa*, whose genome contains a 12.21 % CpG frequency, elicits a higher IL-8 release from HEK-TLR9 cells than the 3.50 % CpG-containing genomic DNA from the commensal bacterium, *Enterococcus faecalis* (Dalpke *et al.*, 2006).

Hemmi *et al.* (2000) demonstrated that TLR-9 confers responsiveness to bacterial DNA in splenocytes, B-cells and macrophages, as their counterparts isolated from TLR-9-

deficient mice were not susceptible to the physiological effects elicited by CpG-DNA. Subsequently, human intestinal epithelial cell lines (HT29, Caco-2 and T84 cells) were shown to constitutively express TLR-9 mRNA and an increased secretion of the proinflammatory cytokine IL-8, from intestinal epithelial cells, was observed in response to CpG-DNA, via a NF- κ B-independent pathway (Akhtar *et al.*, 2003). This suggested a divergence from the IL-8 stimulatory pathway previously characterised in macrophage cells (Chuang *et al.*, 2002). In stark contrast to the system previously reported in mammalian macrophage (where TLR-9 was found to subsist intracellularly, as the immunostimulatory action of CpG-DNA was markedly increased with intracellular delivery of the DNA (Dalpke *et al.*, 2006)), Ewaschuk *et al.* (2007) described an up-regulation of apical surface expression of TLR-9 in intestinal epithelial cells in response to pathogenic bacterial DNA. Dalpke *et al.* (2006) suggested that stimulation of TLR-9 would be difficult in *in vivo* infections, however, their work was undertaken utilising the macrophage model, therefore, only intracellular TLR-9 was considered. Surface expression of TLR-9 in intestinal epithelial cells presents a very real possibility for bacterial DNA to play a role in epithelial immunity.

1.5.8 Flagellin

Flagellin is the highly antigenic, monomeric subunit of bacterial flagella (Ramos *et al.*, 2004). Flagella are rotary motor-like structures, which are expressed by the majority of motile bacteria in the intestine (Berg, 2003). Hayashi *et al.* (2001) determined that bacterial flagella possess Toll-like receptor (TLR)-5 stimulatory ability, and it was confirmed shortly afterwards that TLR-5 exclusively confers cellular responsiveness to extracellular, flagellin (Gewirtz *et al.*, 2001). Monomeric flagellin is naturally released by bacteria, either by leakage due to uncapping or by active depolymerisation (Ramos *et al.*, 2004); however, it can

also be sheared from the bacterial surface by host proteases or detergents (Ramos *et al.*, 2004).

Flagellin plays an important and complex role in intestine homeostasis, having been implicated both as a major antigen in Crohn's disease (Lodes *et al.*, 2004) and as a regulatory moiety in immune responses to commensal bacteria, protecting against spontaneous colitis (Vijay-Kumar *et al.*, 2007). Flagellins from both pathogenic and non-pathogenic bacteria have the potential to stimulate an immune response from the intestinal epithelial layer (Streiner *et al.*, 2000). However, Gewirtz *et al.* (2001) demonstrated that to be able to do this, the flagellin must be translocated from the apical to the basolateral domain of the epithelial layer, despite TLR-5 exhibiting both basolateral *and* apical expression (Cario and Podolsky, 2000). A significant level of translocation is normally considered a trait of pathogenic bacteria (Ljungdahl *et al.*, 2000); therefore the intestinal epithelium is able to distinguish between commensal and pathogenic flagellins by the physical exclusion of commensal bacteria. Epithelial responses to commercially available flagellin (isolated from *Salmonella typhimurium*) have been well characterised with intestinal epithelial cell lines, HT29 and Caco-2 (Bannon, 2008).

1.5.9 Exopolysaccharides

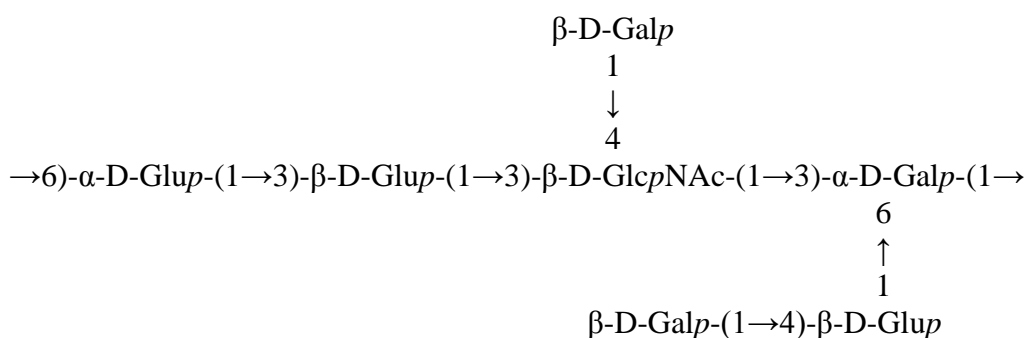
Extracellular polysaccharides, or exopolysaccharides (EPSs), are long-chain polysaccharides released by *Archaeobacteria* and *Eubacteria* (both Gram-positive and Gram-negative) during growth (Badel *et al.*, 2010). EPSs exist in two forms; as membrane-bound moieties, termed capsular polysaccharides (CPSs), or as unbound polymers which exist freely in the extracellular media (Ruas-Madiedo *et al.*, 2002). This section focuses on the free form of EPSs.

EPSs are separated into two categories; homosaccharides and heterosaccharides (Laws *et al.*, 2001). Homosaccharides, such as cellulose, dextran and levan, are made up of only one type of monosaccharide (Laws *et al.*, 2001), and are all extracellularly synthesised by glucansucrase enzymes (Badel *et al.*, 2010). Glucansucrases degrade sucrose from the surrounding media and catalyse the formation of glycosidic bonds between monosaccharides, to form polysaccharide chains (Badel *et al.*, 2010). Conversely, heterosaccharides consist of multiple repeats of oligosaccharides, which themselves are comprised of 3-7 sugar residues (Figure 1.6) (Laws *et al.*, 2001). These oligosaccharide precursors typically contain D-glucose, D-galactose and L-rhamnose sugars (De Vuyst and Degeest, 1999) and occasionally include amino-sugars, such as *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine (Badel *et al.*, 2010). The oligosaccharide subunits of heterosaccharides are constructed intracellularly, by glycosyltransferase enzymes, from sugar nucleotides taken from the surrounding media (Badel *et al.*, 2010). The oligosaccharides translocate out from the bacterial cell and are subsequently polymerised (Ruas-Madiedo *et al.*, 2002). Heterosaccharides are mainly produced by mesophilic and thermophilic bacteria such as lactic acid bacteria (LAB) (De Vuyst and Degeest, 1999) and bifidobacteria (Ruas-Madiedo *et al.*, 2006b, 2010).

1.5.9.1 EPSs in bacterial biofilms

It has been suggested that EPSs play a major role in biofilm formation, and their importance in the initial surface attachment is highlighted by the fact that EPS-deficient mutants completely lack the ability to form biofilms *in vitro* (Watnick and Kolter, 1999). Also, EPSs are likely to stabilise the 3-D structure of biofilms, enabling them to withstand shear forces (Sutherland, 2001). It is theorised that EPSs confer this stability through the minimisation of intercellular repulsions between bacteria by shielding the electrostatic charges on their

A)



B)

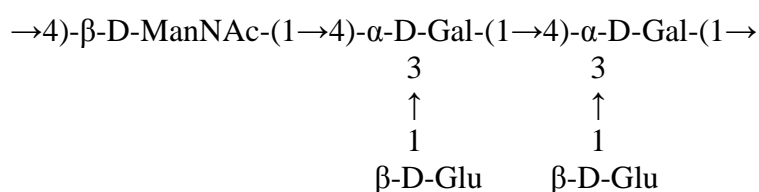


Figure 1.6 – Repeating units of EPSs from *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski. *L. acidophilus* 5e2 EPS consists of a heptasaccharide repeat unit of D-glucose (Glu), D-galactose (Gal) and *N*-acetyl-D-glucosamine (GlcNAc) in the molar ratio 3:3:1 (A), whereas *L. helveticus* sp. Rosyjski EPS constitutes a pentasaccharide repeat unit of D-glucose (Glu), D-galactose (Gal) and *N*-acetyl-D-mannosamine (ManNAc) in the molar ratio 2:2:1 (B). Structures taken from Laws *et al.* (2008) and Leivers (2011), respectively.

surfaces (Watnick and Kolter, 1999). Although EPSs do not exist alone within biofilms, asvarious proteins, lipids and other polysaccharides are also present, it is noteworthy that excessive EPS production by one strain of bacteria can significantly enhance the stability of biofilms of other, non-EPS-producing bacteria (Sutherland, 2001). In addition to astructural role in biofilms, EPSs are thought to be responsible for the enhancement of bacterial nutrient and water entrapping ability (Poulsen, 1999). Furthermore, EPSs are thought to play a key role in protection of biofilm-contained bacteria and have been shown to shield against bacteriophages, antibiotics, lysozyme enzymes and metal ions (Looijesteijn *et al.*, 2001; Durlu-Ozkaya *et al.*, 2007).

However, in stark contrast to the evidence presented above, a study by Kim and colleagues demonstrated a significant anti-biofilm activity of EPS isolated from *Lactobacillus acidophilus* A4, against a range of Gram-positive and Gram-negative pathogenic bacteria (Kim *et al.*, 2009).

1.5.9.2 EPSs in the food industry

Lactic acid bacteria (LAB) are commonly utilised by the food industry for their preservative effects, as their metabolism of sugars releases lactic acid which restricts further microbial contamination (Laws *et al.*, 2001). Also, LAB starter cultures have been used for generations in the production of traditional fermented food goods, such as the Scandinavian milk drink, Kefir, which is produced by the fermentative action of LAB, acetic acid bacteria and yeasts trapped in a complex of EPS and protein (kefir grains) (Vinderola *et al.*, 2006). The Finnish fermented milk drink, viili, also uses a similar process (Ruas-Madiedo *et al.*, 2006a).

LAB are well characterised in their production of EPSs (Cerning, 1990), with over 30 EPS-producing strains described to date (Badel *et al.*, 2010). EPS-producing LAB starter cultures are used in the maturation of cheeses, with the moisture retention of EPSs thought to significantly contribute to the freshness of the cheese (Bhaskaracharya and Shah, 2000). Also, the use of LAB EPSs themselves is increasingly widespread in food products, with EPSs used as biothickening agents in yoghurts, to improve the texture, ‘mouth-feel’ and stability (Marshall and Rawson, 1999; De Vuyst and Degeest, 1999). However, the consequential increase in viscosity from EPSs can also have a negative impact on food goods, with their secretion, by LAB starter cultures, during the production of wine and cider being implicated in the spoiling of these products (de Nadra and de Saad, 1995; Duenas *et al.*, 1995).

1.5.9.3 Prebiotic potential of EPSs

It has been theorised that EPSs are unlikely to serve as a bacterial nutrient reserve, as most EPS-producing bacteria are unable to catabolise their own EPSs (Cerning, 1990; Looijesteijn *et al.*, 2001). However, there is evidence that EPSs could possess prebiotic tendencies. This was first proposed by Ruijsenaars *et al.* (2000) when they tested the biodegradability of food-grade EPSs by human faecal bacteria. Their study demonstrated that mixed bacterial cultures could indeed degrade these EPSs. An investigation by Korakli *et al.* (2002) developed the concept, reporting that EPS isolated from *Lactobacillus sanfranciscensis* could be metabolised by several monocultures of bifidobacteria strains, thus promoting their growth. Nevertheless, the strongest case for the prebiotic potential of EPSs was presented by Salazar *et al.* (2008; 2009), who showed that EPSs isolated from enteric bifidobacteria increased the overall population of *in vitro* faecal bacteria cultures. Additionally, their studies showed an obvious shift in the microbial composition of the cultures, promoting growth of beneficial commensal genera such as bifidobacteria, enterococci and *Bacteroides*, thus satisfying a major stipulation of prebiotics set by Gibson and Rotherford (1995).

Gibson and Roderfroid (1995) also hypothesised that the best prospective prebiotic candidates were non-digestible carbohydrates, such as inulins and fructooligosaccharides, which are able to arrive in the colon intact (Gibson and Roberfroid, 1995). Therefore, for EPSs to function as prebiotics, they must initially be able to survive the mechanical and enzymatic rigours of the GI tract. There has been mixed success in this area of research, with some EPSs, isolated from *Lactococcus lactis* and strains of bifidobacteria, seen to be resistant to gastric enzymes and simulated gastric juices (Looijesteijn *et al.*, 2001; Salazar *et al.*, 2009), while other EPSs, isolated from *Streptococcus thermophilus* and *Lactobacillus casei*, were shown to undergo partial degradation (Mozzi *et al.*, 2009).

1.5.9.4 Physiological influences of EPSs

Kefiran, an EPS produced by a number of strains of lactobacilli in the fermented milk drink Kefir, has been shown to possess a number of systemic physiological activities; these include wound-healing properties, reduction of blood pressure and cholesterol levels, and the retardation of tumour growth in experimental models (Vinderola *et al.*, 2006). Kefiran also exhibits a potential role in intestinal homeostasis, stimulating increased release of lumenal IgA and pro- and anti-inflammatory cytokines, such as IFN- γ , TNF- α , IL-6 and IL-10, observed in the small and large intestine (Vinderola *et al.*, 2006). Additionally, murine macrophages challenged with various EPSs, isolated from strains of lactobacilli and bifidobacteria, demonstrate augmented release of both pro- and anti-inflammatory cytokines, such as TNF- α , IL-6 and IL-10 (Chabot *et al.*, 2001; Bleau *et al.*, 2009; Wu *et al.*, 2010). The mitogenic activity of EPSs isolated from strains of lactobacilli and bifidobacteria is also well characterised, with studies showing the promotion of human, murine, porcine and bovine macrophage proliferation (Kitazawa *et al.*, 1998; Chabot *et al.*, 2001; Wu *et al.*, 2010).

With the high proportion of EPS-producing bacteria naturally residing in the intestine, it is surprising that very little research has been undertaken regarding the interaction of EPSs with the intestinal epithelial layer itself. EPSs, from lactobacilli and bifidobacteria, have previously shown antiproliferative and anticytotoxic activities with bacterial toxin-challenged *in vitro* intestinal epithelial cell (IEC) lines (Ruas-Madiedo *et al.*, 2010; Liu *et al.*, 2011), however, the immunostimulatory effects of EPSs on IECs is a relatively novel concept. To the author's knowledge, the first study to consider this was undertaken by Lebeer *et al.* (2012); however, it must be noted that this was a very minor component of their investigation. Their results demonstrated that EPS isolated from the probiotic *L. rhamnosus* GG had no significant effect on IL-8 mRNA expression in Caco-2 cells. Conversely, a recent review article by Hidalgo-Cantabrana *et al.* (2012) showed preliminary data in which EPS-

producing strains of bifidobacteria were seen to differentially modulate the secretion of inflammatory cytokines, including IL-8, in the Caco-2 intestinal epithelial cell line.

The evidence presented above confirms that EPSs directly associate with host cells in the intestine, however, the molecular mechanisms by which they interact with these cells is, as yet, unknown. Chabot *et al.* (2001) suggested EPSs could exert their action via the mannose receptor. Additionally, a recent study by Ciszek-Lenda *et al.* (2011) demonstrated a cross-tolerance between LPS and EPSs in macrophages, with LPS ‘priming’ of cells lowering the TNF- α release in subsequent EPS challenges, indicating the possibility of a prospective TLR-4 pathway. However, an elegant study by Lin *et al.* (2011) provides the strongest candidate for an EPS receptor. Their investigation identified a novel EPS, TA-1, from the thermophilic marine bacterium, *Thermus aquaticus* and, subsequently, they showed the release of proinflammatory cytokines, TNF- α and IL-6, from murine macrophages in response to challenge with this EPS (Lin *et al.*, 2011). They then utilised the TLR-deficient human embryonic kidney cell line, HEK293T, and its subsequent transfection with various TLRs, to determine which, if any, TLR confers responsiveness to TA-1 EPS, and TLR-2 was considered a possibility. Lin and colleagues then used anti-TLR-2 antibodies on murine macrophages prior to stimulation with TA-1 EPS, and subsequently observed a significant decrease in TNF- α and IL-6 release (Lin *et al.*, 2011). These results strongly suggest that TLR-2 confers EPS responsiveness. This is consistent with the fact that TLR-2 is well characterised in its interactions with a highly diverse range of microbial components (Takeda *et al.*, 2003; Akira *et al.*, 2006).

1.5.10 Exotoxins and enterotoxins

Pathogenic bacteria have evolved numerous methods for evading the host’s defences and for disrupting the functionality of host cells; for example, toxin production (Balfanz *et al.*, 1996).

In microbiological terms, ‘toxins’ are products of microbial metabolism that have a detrimental effect on host cell function (Balfanz *et al.*, 1996). Proliferating pathogenic bacteria often produce extracellular toxin (exotoxin) proteins, which are generally secreted into the surrounding environment at a site of infection, however, they can also cause damage at distant sites (Madigan and Martinko, 2006). Exotoxins can be separated into 3 distinct categories: membrane-damaging, receptor-targeting and internalising toxins (Balfanz *et al.*, 1996). The main effect of membrane-damaging toxins is disruption of the cell membrane, leading to increased permeability and cell death by lysis (Balfanz *et al.*, 1996). Receptor-targeting toxins target specific physiological surface receptors and modulate the resultant intracellular reaction cascades (Balfanz *et al.*, 1996). Internalising toxins require the process of receptor binding and internalisation by endocytosis before they can implement their intracellular toxic effect (Balfanz *et al.*, 1996).

Enterotoxins are a class of exotoxins which are specific in their action in the intestine (Binder and Powell, 1970). Their modes of action vary, but can generally be separated into two categories; cytotoxic, which cause cell damage and physically disrupt the epithelial layer; or cytotoxic, which function to induce active chloride ion (Cl^-) secretion and inhibit sodium ion (Na^+) absorption, to, and from, the intestinal lumen, by enterocytes (Farthing, 2000). The interference in barrier function or ion transfer results in excessive fluid secretion into the intestinal lumen, generally leading to diarrhoea (Madigan and Martinko, 2006).

1.5.10.1 *C. difficile* and its enterotoxins

Clostridium difficile is a spore-forming, anaerobic, Gram-positive pathogen implicated as the main causative agent in pseudomembranous colitis (PMC) (Davies *et al.*, 2011, Canny *et al.*, 2006). PMC is characterised by inflammation of the colonic mucosa and the formation of a ‘pseudomembrane’ (comprised of sloughed epithelial cells, mucin and polymorphonuclear

cells) which covers the mucosal layer (Mahida *et al.*, 1996). Furthermore, *C. difficile* is responsible for 15-20 % of all cases of antibiotic-associated diarrhoea (AAD) (Genth *et al.*, 2008, Janvilisri *et al.*, 2010) and can lead to toxic megacolon, shock and, in severe cases, death (Clements *et al.*, 2010). The virulence of *C. difficile* stems mainly from its two major cytotoxic enterotoxins; toxin A (TcdA) and toxin B (TcdB) (Carter *et al.*, 2011, Lica *et al.*, 2011, Zemljic *et al.*, 2010). However, some strains also produce a binary toxin, CDT, which has also demonstrated independent cytotoxic activity (Sundriyal *et al.*, 2010).

TcdA and TcdB are monoglucosyltransferases which intracellularly inactivate Rho family guanosine triphosphate hydrolyses (GTPases), such as Rho, Ras, Ral and Cdc42 (Carter *et al.*, 2011). Rho GTPases are molecular switches which control the regulation of the actin cytoskeleton within epithelial cells (Davies *et al.*, 2011). However, before the toxins can implement their activity, they must first gain access to the host cell cytoplasm. The process initiates with TcdA or TcdB binding to unknown, non-proteinaceous cell surface receptors (Voth and Ballard, 2005). Receptor-binding triggers endocytosis of the toxins and, once internalised, the endosome acidifies due to an influx of hydrogen (H^+) ions (Voth and Ballard, 2005). The decrease in pH stimulates conformational changes in the toxins, exposing their hydrophilic domains and allowing their insertion into the endosomal membrane (Voth and Ballard, 2005). The resultant pore in the endosomal membrane allows the glucosyltransferase domain to migrate to the cytosol where the Rho GTPases reside (Genth *et al.*, 2008). The glucosylating activity of both TcdA and TcdB causes the degradation of filamentous (F-) actin to the monomeric conformation (G-actin) and, at a cellular level, this leads to changes in cell morphology (cell rounding) and loosening of intercellular tight junctions (Nusrat *et al.*, 2001). The consequences of TcdA and TcdB at the organ level include increased epithelial permeability and augmented epithelial production of cytokines

and chemokines, which in turn triggers neutrophil infiltration, mast cell activation and direct damage to the intestinal mucosa (Voth and Ballard, 2005).

C. difficile binary toxin, CDT, is an AB-toxin consisting of the enzymatically active A-subunit, CDTa, and the catalytically inert transport component, the B-subunit, CDTb (Davies *et al.*, 2011). CDTb delivers CDTa to the host cell cytoplasm via receptor-mediated endocytosis (Barth *et al.*, 2004). However, to achieve this CDTb must first be proteolytically cleaved from CDTa, activating it and allowing it to form heptamers which then bind specific cell surface receptors (Barth *et al.*, 2004). CDTa re-binds to the transporter domain and undergoes endocytosis. Once internalised, the endosome acidifies and CDTb forms a pore in the endosomal membrane, delivering CDTa to the cytosol (Barth *et al.*, 2004). The CDTa subunit is an actin-ADP-ribosylating toxin which acts to transfer a ribose moiety from nicotinamide adenine dinucleotide (NAD), or its oxidised form, NADH, to monomeric (G-) actin, thus preventing polymerisation to the filamentous configuration (F-actin) (Sundriyal *et al.*, 2010). The role of CDT in the pathogenesis of *C. difficile* infection is yet to be established, however, it has been implicated in TcdA- and TcdB-independent cytotoxic activity (Sundriyal *et al.*, 2010), thus suggesting a role in the aetiology of *C. difficile* infection.

1.6 Project aims and objectives

It is apparent that enteric bacteria are a vast potential source of a wide range of antigenic products; however, under normal physiological conditions, the intestinal mucosal immunity remains hyporesponsive, yet, it also retains the innate ability to rapidly respond to pathogens and their respective antigens. Despite this, the specific immunomodulatory activities of the extracellular products from non-pathogenic, enteric-associated bacteria have been little

studied, as previous investigations have been heavily reliant on pathogenic-derived antigens to study epithelial interactions.

Specific objectives of the current project were to:

- a) Characterise and compare the two *in vitro* intestinal epithelial cell lines HT29-19A and Caco-2 in their suitability for modelling epithelial interactions with bacterial extracellular products.
- b) Investigate the inflammatory properties of the extracellular products of the Gram-negative commensal *Escherichia coli* C25.
- c) Examine the inflammatory activities of extracellular products, specifically ultrapure EPSs, of the previously unstudied *Lactobacillus acidophilus* 5e2 and *Lactobacillus helveticus* sp. Rosyjski.
- d) Investigate possible protective effects of non-pathogenic extracellular products against those from the enteropathogenic bacterium *C. difficile*.

Chapter 2

Materials and methods

2.1 Materials

Cell culture media ingredients, supplements and related solutions were all purchased from Gibco® Invitrogen™ Life Technologies Ltd., Paisley, UK, with the exception of fetal bovine serum (FBS) and 0.25x trypsin/EDTA solution which were sourced from Biosera Ltd., East Sussex, UK and Sigma-Aldrich Company Ltd., Dorset, UK, respectively. All 25 and 75 cm² cell culture flasks, 12- and 24-well culture plates and 35 x 10 mm and 100 x 20 mm culture dishes were from Cellstar® Greiner Bio-One Ltd., Stonehouse, UK. 96-well cell culture plates were purchased from Matrix®, Thermo Fisher Scientific UK Ltd., Loughborough, UK. HT29-19A (Augeron and Laboisie, 1984) and Caco-2 (ATCC No. HTB-37) cell lines were kindly donated by Prof. G. Warhurst, Salford Hope Hospital, Royal NHS Foundation Trust & University of Salford, UK. All bacterial agar and broth powders were purchased from Lab M Ltd., Bury, UK. All bacterial strains were kindly provided by Dr. P. Humphreys, Department of Chemical and Biological Sciences, University of Huddersfield, UK. Ultrapure lactobacilli EPSs were kindly supplied by Prof. A. Laws, Department of Chemical and Biological Sciences, University of Huddersfield, UK. 96-well ELISA-treated microplates were purchased from Greiner Bio-One Ltd., Stonehouse, UK. IL-8 and IL-10 Human Antibody Pairs and stabilised chromagen (tetramethylbenzidine (TMB)) solution were purchased from Invitrogen, Paisley, UK. The DC Protein Assay kit was purchased from Bio-Rad Laboratories Ltd., Hemel Hempstead, UK. The Ultraclean® Soil DNA Isolation Kit and LPS Extraction Kit were both from Chembio Ltd., Hertfordshire, UK. The QIAquick® PCR Purification Kit, RNeasy® Mini Kit and RNase-free DNase Set were all from Qiagen, Crawley, UK. The iScript™ cDNA Synthesis Kit was purchased from Bio-Rad Laboratories Ltd., Hemel Hempstead, UK. All PCR primers used were purchased from Eurofins MWG Operon, Ebersberg, Germany. Universal ProbeLibrary probes and Lightcycler® Taqman® Mastermix were purchased from Roche Diagnostics Ltd., West Sussex, UK. REDTaq® ReadyMix™ PCR

Reaction Mix was purchased from Sigma Aldrich Company Ltd., Dorset, UK. Peptidoglycan isolated from *Bacillus subtilis*, lipopolysaccharide from *E. coli* 0111:B4 and deoxyribonucleic acid (DNA) sodium salt from *Escherichia coli* strain B were purchased from Sigma Aldrich. Flagellin from *Salmonella typhimurium* strain 14028 was from Enzo Life Sciences Ltd., Exeter, UK. DNA sequencing was undertaken by Eurofins MWG Operon, Ebersberg, Germany. All other reagents were purchased from Sigma Aldrich.

2.2 Culturing of intestinal epithelial cells

2.2.1 HT29-19A and Caco-2

The HT29-19A (passages 41-70) and Caco-2 (passages 39-68) cell lines were grown in a media of high glucose (4500mg/l) Dulbecco's Modified Eagles Media (DMEM), 10 % foetal bovine serum (FBS), 4 mM glutamine and a mixture of 50 IU/ml penicillin and 50 µg/ml streptomycin (PenStrep). In addition to this, HT29-19A cells were supplemented with 20 mM HEPES solution and Caco-2 cells had 0.1 mM MEM NEAA (non-essential amino acids) added. The cell lines were incubated at 37 °C, with 5 % carbon dioxide (CO₂) and constant humidity, and the media was replaced every 2-3 days.

2.2.2 Freezing/thawing cell lines

In preparation for freezing, cells were fed 24 h previously. Cells were trypsinized and incubated at 37 °C to detach them from the surface of the culture flask. 0.05% trypsin/EDTA solution was deactivated by the addition of fresh culture media. The cell suspension was centrifuged (1000 g for 3 min) and the supernatant was removed. The cell pellet was resuspended in cold cell culture media (supplemented with 10 % DMSO (dimethyl sulphoxide) and 20 % FBS) at a density of 3×10^6 cells per cryo-vial. The cryo-vials were placed in a 'Mr Frosty' freezing container at -80 °C overnight and were transferred to a

liquid nitrogen store (-176 °C) until required. The 'Mr Frosty' contained isopropanol, which cools at 1 °C/min, thus avoiding the formation of ice crystals, and subsequent cell lysis.

DMSO is toxic to the cells at 4 °C+, therefore, rapid thawing of the cells was required to avoid toxicity. Cryo-vials were quickly thawed by placing in a 37 °C water bath. Once cells were completely thawed, cold cell culture media was added, cell suspensions were centrifuged (1000 g for 3 min) and the supernatant was removed. The pellet was resuspended in fresh media and transferred to a 25 cm² culture flask, which was incubated at 37 °C, 5 % CO₂ and constant humidity. Once confluent, the entire cell population of the 25 cm² flask was transferred to a 75 cm² flask and normal passage procedure was followed.

2.2.3 Cell passage

Cell lines were sub-cultured to 80 % confluence or greater in 75 cm² culture flasks, at which point they were transferred to new culture flasks or plates. The spent media was removed and cells were washed 2-3 times with Hank's Buffered Salt Solution (HBSS) (-Mg²⁺ and -Ca²⁺) to remove any traces of serum. Once the cells had been washed, they were exposed to 0.05% trypsin/EDTA solution at 37 °C for 5–10 min, or until all the cells had completely detached from the surface of the flask. The trypsin was subsequently deactivated with the addition of fresh cell culture media (containing 10 % FBS). The resultant cell suspension was homogenised with repeated pipetting, spilt into a relevant number of aliquots and transferred to new culture flasks or plates with fresh media. Continuation flasks were normally seeded at a 1 in 8 dilution to give a confluent flask after incubation for 7 days.

2.2.4 Seeding cell culture plates

For experimental work HT29-19A and Caco-2 cells were seeded on 12- and 24-well cell culture plates at a cell density of 0.5 x 10⁵ cells/cm², however, 96-well plates were seeded at

0.5×10^3 cells/cm². The cells were grown to 100 % confluence over 7 days and cell culture media was replaced every 2-3 days.

2.2.5 Cell counting/viability

Cells were removed from the surface of the culture plate with the use of a cell scraper (Thermo Fisher Scientific UK Ltd., Loughborough, UK). The resultant cell suspension was aspirated off and the plate was washed with fresh media, to ensure all cells were removed. The total volume of the cell suspension was recorded, the cell suspension was homogenised by repeated pipetting and an aliquot was taken and added in a 1:1 ratio with trypan blue dye. Trypan blue distinguishes viable and non-viable cells by penetrating the cell membrane of dead cells and staining the cell contents blue, whereas viable cells do not absorb the dye. An Improved Neubauer haemocytometer (Thermo Fisher Scientific UK Ltd., Loughborough, UK) was used to count the cells. The average cell counts, for both dead and alive cells, from the four corner squares on the haemocytometer were taken and the resultant cell count was multiplied by 2 (for the dilution by the trypan blue) and then by 10^4 , to calculate the cell number per millilitre of cell suspension. Cell viability was then calculated. The expected viability from a 'normal' culture plate was 85-100 %.

2.2.6 Dome counting

Caco-2 cells were grown to confluence (7 days) on 24-well cell culture plates. The domes were quantified in the wells of the culture plate by counting the number of domes in ten low power fields of view (100x magnification), taking an average and then multiplying by the number of fields of view equivalent to the surface area of the wells (in this case 56.37). Domes were identified as small circular patches of out-of-focus cells (due to sitting on a different geometrical plane to the rest of the plate of cells), as shown in Figure 2.1.

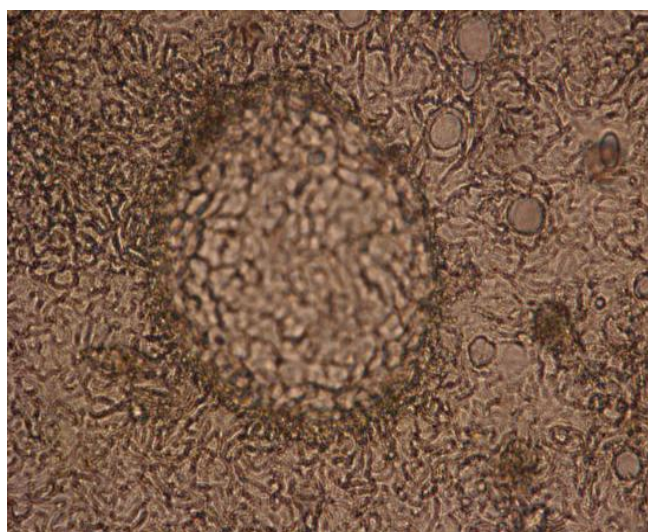


Figure 2.1 – Dome structures on Caco-2 monolayers. Domes exist in a different geometric plane to the monolayer and so appear out-of-focus.

2.2.7 Cell challenge experiments

Epithelial cells were fed with fresh cell culture media prior to confluence (6 days in culture) and incubated for 24 h at 37 °C, 5 % CO₂ and constant humidity. Subsequently, confluence was reached and the spent media supernatant was removed. The cells were incubated with the appropriate challenge sample for 24 h at 37 °C, 5 % CO₂ and constant humidity. All challenge samples were serially diluted in standard cell culture media (unless stated that FBS-free media was used) and media alone served as control. The supernatants were collected and frozen at -80 °C until ELISA analysis (see Section 2.8) could be performed. The cell layers were also stored at -80 °C until protein analysis (see Section 2.9) or RNA isolation (see Section 2.10.2) could be performed.

2.3 Bacteria

2.3.1 *Clostridium difficile* cell-free supernatant

C. difficile (NCTC 13366, Ribotype 027) was cultured in tryptone soy broth (TSB) with 5 % lysed horse blood for 14 days in strict anaerobic conditions. Subsequently, the culture was centrifuged at 6000 x g for 10 min, and the supernatant was sequentially filtered using 0.80 µm and 0.22 µm vacuum-driven bottle top filter systems (Nalgene®, Thermo Fisher Scientific UK Ltd., Loughborough, UK and Millipore UK Ltd., Watford, UK, respectively). The cell-free supernatant was serially diluted in cell culture media and used for cell challenge experiments.

2.3.2 *Escherichia coli* C25 cell-free supernatant

E. coli C25 was cultured on tryptone soy agar (TSA) overnight at 37 °C. DMEM, supplemented with 4 mM glutamine was inoculated with *E. coli* C25 and incubated overnight (~18 h), until the culture reached the stationary phase of growth (~1 x 10⁹ CFU/ml). Subsequent to incubation, the culture was centrifuged at 6000 x g for 10 min to pellet out the bacteria. The supernatant was poured off, had its pH returned to 7.4 and was subsequently filtered using a 0.45 µm syringe-driven filter (Millex®, Millipore UK Ltd., Watford, UK.). The cell-free supernatant was serially diluted in cell culture media and used in cell challenge experiments.

2.3.3 *Lactobacilli* cell-free supernatant

Lactobacillus acidophilus 5e2 and *Lactobacillus helveticus* sp. Rosyjski were cultured on de Man, Rogosa and Sharpe (MRS) agar, supplemented with 0.05 % L-cysteine hydrochloride, for 48 h at 37 °C in strict anaerobic conditions. The plate cultures were washed off in to 2 ml maximum recovery diluent (MRD) and used to inoculate 500 ml MRS broth. Resultant broth

cultures were incubated for 24 h at 37 °C in anaerobic conditions. The cultures were centrifuged at 6000 x g for 10 min to pellet out the bacteria and the supernatant was poured off, had its pH adjusted to 7.4 and was filtered through a 0.45 µm syringe-driven filter. The supernatants were serially diluted in cell culture media for cell challenge experiments.

2.3.4 Sonicated bacteria

10 ml aliquots of bacterial cultures were sonicated, using a Vibracell VCX 130 (Sonics and Materials Inc., Newtown, CT, USA) at 85 % amplitude for a 5 x 6 s pulse program. The cultures were sonicated on ice and with a 24 s cooling step between pulses (to prevent denaturation of proteins, DNA etc.). Resultant solutions were filtered through a 0.45 µm syringe-driven filter, serially diluted in cell culture media and used in cell challenge experiments.

2.4 Ultrapure *Lactobacillus* exopolysaccharides

The multistep extraction and ultrapurification of exopolysaccharides (EPSs) from bacterial cultures was described by Marshall and Rawson (1999). Briefly, *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski were cultured in 10% (w/v) skimmed milk solution (St. Ivel Ltd., Swindon, UK) supplemented with 0.166 M glucose for 24 h at 42 °C. The resultant culture was used to inoculate, at 1% (v/v) concentration, a greater volume of skimmed milk solution, supplemented with 0.166 M glucose. The culture was fermented for 48 h at 42 °C. Fermentation was stopped with the addition of 80% (w/v) trichloroacetic acid (TCA), giving a final concentration of 14 % (v/v) of TCA, and the resulting solution was stored overnight at 4 °C. The solution was then centrifuged at 25,000 g (Avanti J-26 XPI centrifuge, Beckman Coulter Ltd UK, High Wycombe, UK) for 35 mins at 4 °C in order to remove bacteria and precipitated proteins. The solution was then filtered through grade 4 filter paper (Whatman

UK Ltd, Kent, UK), and crude EPS was precipitated by the addition of an equal volume of chilled absolute ethanol; this was stored overnight at 4°C. The sample was then centrifuged at 25,000 g for 35 mins at 4°C. The recovered pellet was re-dissolved in deionised water (~50 ml). Gentle heating in a water bath at 50 °C was required for complete dissolution of the pellet. The dissolved crude EPS was subjected to another precipitation step, followed by subsequent centrifugation as described above. The pellet obtained was then re-dissolved in a minimum of deionised water (~10 mL); again gentle heating in a water bath at 50°C was required for complete dissolution of the pellet. Small neutral sugars were then removed by dialysis, for 72 h at 4°C, against three changes of deionised water per day. After three days, the contents of the dialysis tubing was collected in a round-bottom flask and lyophilised using an Edwards freeze-drier (Northern Scientific, York, UK). The dry weight of the EPS produced was then determined. EPS purity was subsequently confirmed by size exclusion chromatography using a multi-angle laser light scattering detector and highly intensive NMR analysis (Leivers, 2011).

2.5 Bacterial outer membrane vesicle isolation

Outer membrane vesicle isolation method was adapted from Kadurugamuwa and Beveridge (1995). 250 ml *E. coli* C25 cultures were centrifuged at 6000 x g for 10 min to pellet out the bacteria. The supernatant was sequentially filtered through 0.80 and 0.45 µm pore size vacuum-driven bottle top filters. The filtrate was ultracentrifuged at 150,000 x g for 1.5 h, at 5 °C, to pellet out the membrane vesicles. The supernatant was removed and the MV pellet was resuspended in 50 mM HEPES buffer (pH 6.8) and ultracentrifuged again for 30 min at 120,000 x g, 5 °C. The supernatant was again removed and the MV pellet was resuspended in 50 mM HEPES buffer (pH 6.8), filtered through a 0.45 µm syringe filter and stored at 4 °C.

2.6 Lipopolysaccharide isolation

The LPS Extraction Kit (Chembio Ltd., Hertfordshire, UK) was used to isolate the lipopolysaccharide (LPS) from gram-negative bacteria. Pre-made lysis and purification buffers were provided with the kit. The protocol provided by the manufacturer was followed. In brief, *E. coli* C25 was cultured in TSB to an OD₅₂₅ of 1.0. Subsequently, 5 ml bacterial culture was centrifuged for 10 min at $\geq 10,000 \times g$, to pellet out the bacteria. The supernatant was removed, 1 ml lysis buffer was added and the mixture was vortexed vigorously until the bacterial pellet disappeared. 200 μ l chloroform was added and the mixture was vortexed for 10-20 s and incubated at room temperature for 5 min. The mixture was centrifuged for 10 min at $\geq 10,000 \times g$, 4 °C, separating it into two distinct layers, a colourless layer on top of a blue layer. 400 μ l of the colourless layer was pipetted off, added to 800 μ l purification buffer and homogenised by repeated pipetting. The mixture was incubated at -20 °C for 10 min and centrifuged for 15 min at $\geq 10,000 \times g$ to pellet out the LPS. The supernatant was removed and the pellet was washed in 1 ml 70 % ethanol. The mixture was centrifuged for 3 min at $\geq 10,000 \times g$ and the supernatant was removed. The LPS pellet was left to dry at room temperature and resuspended in 10 mM Tris-HCl (pH 8.0). A typical 5 ml bacterial culture is estimated to yield ~30 μ g LPS.

2.7 Bacterial translocation assay

The method for this assay was developed from the technique described by Macutkiewicz *et al.* (2008). Epithelial cells were cultured to confluence (7 days) and fed 24 h in advance of the assay. 10 ml sterile tryptone soy broth (TSB) was inoculated with *E. coli* C25 and incubated at 37 °C overnight (18 h), giving a culture in the stationary phase of growth and at a density of $\sim 1 \times 10^9$ CFU/ml (serial dilutions and plating out in to tryptone soy agar (TSA) gave exact numbers). The culture was centrifuged at $10,000 \times g$ for 10 min and the bacterial

pellet was resuspended in 10 ml HBSS supplemented with 10 mM sodium bicarbonate and 180 mg/dl glucose (Trans-HBSS). Trans-HBSS reduces growth of bacteria 1000-fold when compared to bacterial growth in cell culture media (Clark *et al.*, 2003). Epithelial cells were washed twice in sterile Trans-HBSS, had $\sim 1 \times 10^9$ CFU C25 in 2 ml Trans-HBSS added and were incubated at 37 °C for 4 h. After incubation, the supernatant was removed, serially diluted to 10^{-6} and 10^{-7} dilutions and plated out in TSA to give an indication of bacterial growth during the incubation period. Epithelial cells were again washed twice with Trans-HBSS, thus removing any bacteria not adhered to, or been internalised by, the epithelial cells. 1 ml Trans-HBSS/50 µg/ml gentamicin was added to the cells and incubated at room temperature for 15 min, thus killing all but the internalised bacteria (the antibiotic cannot enter the epithelial cells and therefore did not affect the bacteria which had been internalised). To investigate numbers of bacteria which had translocated *and* adhered, the incubation with gentamicin was left out. The supernatant was removed and plated out neat, in TSA, to observe if the antibiotic had killed the extracellular bacteria. The epithelial cells were lysed by osmotic pressure with the addition of 1 ml sterile deionised water, and by sheer force by repeated pipetting, thus releasing the bacteria present inside the cells. A serial dilution of the lysate was performed to give 10^{-3} and 10^{-4} dilutions (10^{-6} and 10^{-7} dilutions in adherence *and* translocation), which were subsequently plated out, in TSA, using the agar pour plate method. All resultant plates were incubated overnight at 37°C and bacterial colonies were observed and quantified.

2.8 ELISA analysis

2.8.1 Interleukin (IL)-8

The concentration of IL-8 released in to the culture media by epithelial cells was quantified using a commercial IL-8 enzyme-linked immunosorbant assay (ELISA) antibody pair

(BioSource™, Invitrogen, Paisley, UK). Coating buffer A, washing buffer and assay buffer were all made up to manufacturer's specifications. 96-well microplates (Greiner Bio-One Ltd., Stonehouse, UK) were coated with the human IL-8-specific coating antibody (1 µg/ml), which had previously been diluted in coating buffer A, for 18 h at 4 °C. The wells of the pre-coated microplate were aspirated and rinsed with washing buffer (3x). Blocking was undertaken by addition of 400 µl assay buffer in to each well, and the plate was subsequently incubated at room temperature for 1 h. The lyophilised IL-8 standard was reconstituted in ultrapure water and serially diluted to give concentrations of 800, 400, 200, 100, 50, 25 and 12.5 pg/ml. Subsequent to blocking, 100 µl of the standards and a blank (0 pg/ml of IL-8) were added, in duplicate, into appropriate wells. 100 µl of the samples being assayed were also loaded into the relevant wells. 50 µl detection antibody was immediately added to the wells and the plate was incubated at room temperature, for 2 h, on a shaker bed set to 500-700 rpm. The plate was washed 3x with washing buffer, after which, 100 µl streptavidin-horse radish peroxidase (HRP), which had previously been diluted to a concentration of 1 in 2500 in assay buffer, was loaded into each well and the plate was incubated for 30 min, at room temperature and on a shaker bed set to 500-700 rpm. Following incubation, the wells were washed three times with washing buffer. 100 µl stabilised chromagen (tetramethylbenzidine (TMB)) solution (Invitrogen, Paisley, UK) was added to each well and the plate was incubated for a final time, at room temperature on a shaker bed set to 500-700 rpm, for 30 min (*Note: with the addition of the TMB, a blue colour was evident*). After incubation, 100 µl stop solution (1 M sulphuric acid) was added to each well, inducing a colour change, from blue to yellow. The absorbance of the resulting mixture was read at 450 nm within 30 min of stopping the reaction. The absorbance value obtained from the ELISA analysis was converted to concentration of IL-8, with the aid of a calibration curve (Figure

2.2). In all experiments IL-8 concentration was normalised and subsequently expressed as pg IL-8 per mg of total protein present on the cell culture plate (pg/mg total protein).

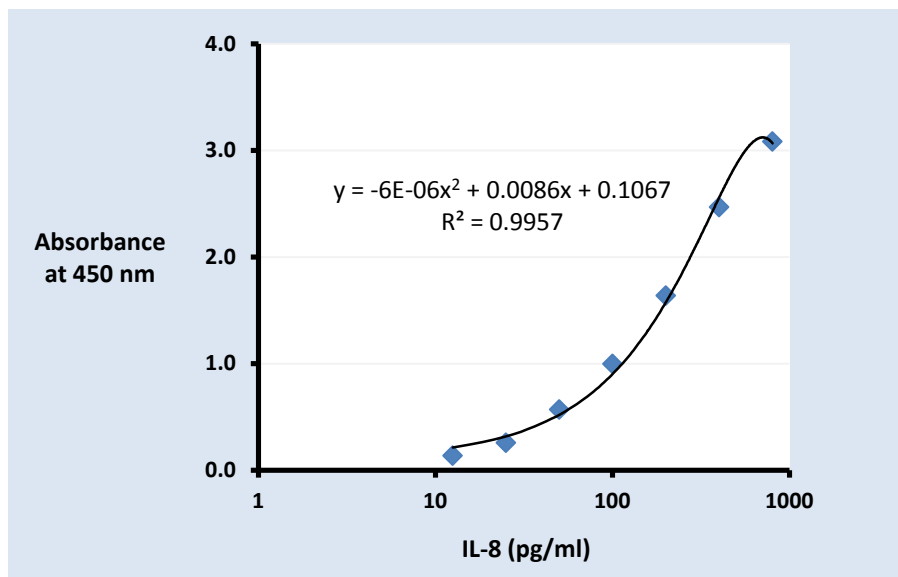


Figure 2.2 – A typical IL-8 ELISA standard curve. Lyophilised IL-8 standard (provided with the ELISA kit) was reconstituted to a concentration of 800 pg/ml. This was serially diluted and ELISA analysis was undertaken. Data shown is mean of duplicates of each concentration.

2.8.2 Interleukin (IL)-10

IL-10 released by epithelial cells was quantified using a commercial IL-10 ELISA antibody pair (BioSource™, Invitrogen, Paisley, UK). The same protocol was used as for the IL-8 ELISA assay, with the exception of a different antibody pair and different serial dilutions of the lyophilised standard. The IL-10 standard was resuspended and serially diluted in ultrapure water to concentrations of 2000, 1000, 500, 250, 125, 62.5, 31.25 pg/ml. A typical calibration curve is shown in Figure 2.3.

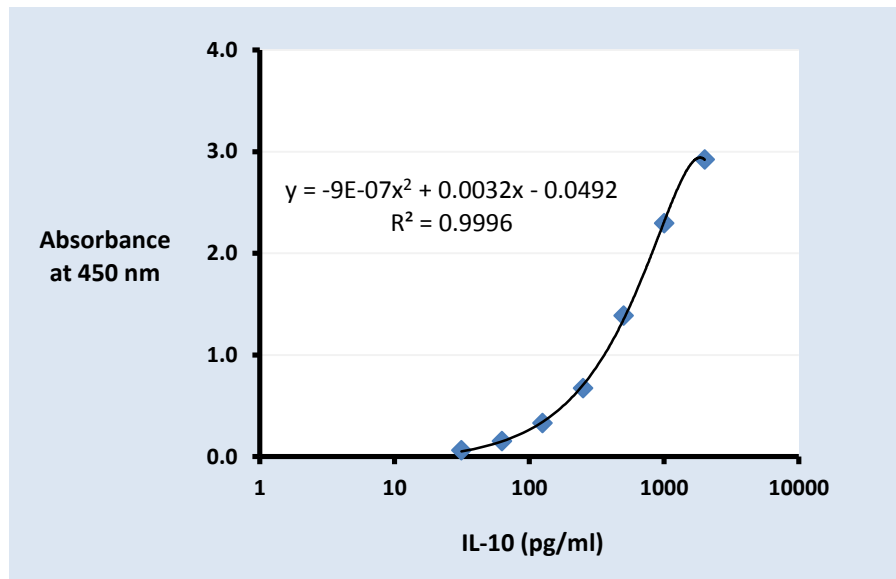


Figure 2.3 – A typical IL-10 ELISA standard curve. Lyophilised IL-10 standard (provided with the ELISA kit) was reconstituted to a concentration of 2000 pg/ml. This was serially diluted and ELISA analysis was undertaken. Data shown is mean of duplicates of each concentration.

2.9 Protein analysis

The *DC* Protein Assay (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), a technique based on the Lowry assay (Lowry *et al.*, 1951), was used to estimate the protein content of layers of epithelial cells. The resultant absorbance gained from the assay was compared to a standard curve produced from bovine serum albumin (BSA) and converted to protein concentration (mg/ml). The standard curve was prepared using serial dilutions of BSA resuspended in ultrapure water at concentrations ranging from 0-1.5 mg/ml.

Prior to protein analysis, epithelial cells were lysed by addition of 1 ml sterile water. 100 µl aliquot of the lysate was added to 500 µl Reagent A (an alkaline copper tartrate solution). The mixture was vortexed and 4 ml Reagent B (the Folin reagent) was added to the mixture. The copper tartrate, which reacts with the peptide bonds present in the protein suspension, reduces the Folin reagent, inducing a colour change from yellow to blue. The depth of the blue is representative of the number of peptide bonds, and therefore the amount

of protein, present in the suspension. The absorbance of the mixture was measured at 750 nm and this was converted, using the BSA standard curve, to a concentration of protein in the suspension (mg/ml).

2.10 DNA/RNA isolation and analysis

2.10.1 DNA isolation from bacteria

The Ultraclean® Soil DNA Isolation Kit (Cambio Ltd., Cambridge, UK) was used to isolate the DNA from the bacteria. All solutions were made up to the manufacturer's specifications and all consumables were provided with the kit. An inoculating loopful of bacteria was added to the 2 ml bead solution tubes provided with the kit and the mixture was briefly vortexed. 60 µl solution S1 was added and the tube was vortexed again. 200 µl inhibitor removal solution (IRS) was added and the bead solution tube was secured horizontally on a vortex with the Mo Bio Vortex Adapter tube holder (Cambio Ltd., Cambridge UK). The tube was vortexed at maximum speed for 10 min. Subsequent to vortexing, the tube was centrifuged at 10,000 x *g* for 30 s. The supernatant was transferred to a 2 ml collection tube and 250 µl solution S2 was added. The mixture was vortexed for 5 s and incubated at 4 °C for 5 min. The mixture was centrifuged for 1 min at 10,000 x *g* and the supernatant was transferred to a clean 2 ml collection tube. 1.3 ml solution S3 was added and the mixture was vortexed for 5 s. 700 µl of mixture was loaded on to a spin filter column and centrifuged at 10,000 x *g* for 1 min. The flow through was discarded and the process was repeated until all the sample has passed through the filter. 300 µl solution S4 was added to the filter and was centrifuged at 10,000 x *g* for 30 s. The flow through was discarded and the spin column was centrifuged again at 10,000 x *g* for 1 min. The spin column was transferred to a clean 2 ml collection tube and 50 µl solution S5 was added to the centre of the filter membrane. The spin column was centrifuged at 10,000 x *g* for 30 s, the DNA eluted and the spin column was discarded. The

ultraviolet (UV) absorbance was measured at 260 and 280 nm to determine purity and concentration of the DNA (Section 2.10.3 and 2.10.4). The bacterial DNA sample was stored at -80 °C until required.

2.10.2 RNA isolation from adherent human cell lines

RNA was isolated using the RNeasy[®] Mini Kit (Qiagen, Crawley, UK) in conjunction with the RNase-free DNase Set (Qiagen, Crawley, UK). All tubes, buffers and solutions (except ethanol) were provided by the manufacturer, as part of the kits. HT29-19A and Caco-2 cells used for RNA extraction were cultured on 35 mm x 100 mm cell culture dishes. Post-challenge, the cell supernatant was aspirated off and the cells were lysed with the addition of 600 µl RLT buffer. The cells were removed from the surface of the plate with a cell scraper and the mixture was homogenised by repeated pipetting. 600 µl 70 % ethanol was added to the homogenate and was mixed by pipetting. 600 µl sample was loaded on to an RNeasy[®] spin column placed inside a 2 ml collection tube and spun at $\geq 8000 \times g$ for 15 s. The flow through was discarded and the remaining 600 µl sample was loaded on to the spin column and centrifugation was repeated. On-column DNA digestion was used to clean the RNA sample of DNA contamination. Prior to DNA digestion, the lyophilized DNase I (1500 Kunitz units) was resuspended in 550 µl of RNase-free water to give a DNase I stock solution. 350 µl Buffer RW1 was added to the spin column, centrifuged at $\geq 8000 \times g$ for 15 s and the flow through was discarded. 10 µl DNase I stock was added to 70 µl Buffer RDD and the mixture was added to the spin column and incubated at room temperature for 15 min. 350 µl Buffer RW1 was added to the spin column and centrifuged at $\geq 8000 \times g$ for 15 s and the flow through was discarded. 500 µl Buffer RPE was added to the spin column and centrifuged $\geq 8000 \times g$ for 2 min. The 2 ml collection tube and flow through were discarded and the spin column was placed inside a new 2 ml collection tube and centrifuged at $\geq 8000 \times g$

g for 1 min. The collection tube and flow through were discarded and replaced by a new 1.5 ml collection tube. 50 µl RNase-free water was added to the spin column and centrifuged at $\geq 8000 \times g$ for 1 min. A further 50 µl RNase-free water was added to the spin column and the centrifugation was repeated. The UV absorbance was measured at 260 and 280 nm to determine purity and concentration of the RNA (Section 2.10.3 and 2.10.4). The RNA sample was stored at -80 °C until required.

2.10.3 DNA/RNA quantification by UV spectrometry

To quantify the DNA/RNA in a sample, the ultraviolet (UV) absorbance was measured at 260 nm (A_{260}). An absorbance of 1 unit at this wavelength corresponds to 44 µg of DNA/RNA per ml, therefore the concentration of DNA/RNA can be calculated by:

$$\text{Concentration of DNA/RNA sample} = 44 \mu\text{g/ml} \times A_{260} \times \text{dilution factor} \quad (\text{Equation 2.1})$$

The amount of RNA in the sample was calculated by:

$$\text{Amount of DNA/RNA} = \text{concentration} \times \text{volume (ml)} \quad (\text{Equation 2.2})$$

2.10.4 DNA/RNA purity

The purity of the DNA/RNA samples was estimated by calculating the ratio of the UV absorbance readings at 260 and 280 nm (A_{260}/A_{280}). This ratio gives purity with respect to contaminants, such as protein and organic compounds, which absorb light in the UV spectrum. Pure DNA gives an $A_{260/280}$ ratio of ~1.8 and pure RNA gives a ratio of ~2.

2.10.5 Isolation and purification of the 16S gene from bacteria for sequencing

The Ultraclean[®] Soil DNA Isolation Kit (Cambio Ltd., Cambridge, UK) is used to isolate the DNA from *E. coli* C25. All solutions are made up to the manufacturer's specifications and all

consumables are provided with the kit. The method was described previously in Section 2.10.1.

The bacterial DNA was subjected to PCR, in order to amplify the 16S ribosomal gene. The following PCR mix was used: 25 µl REDTaq[®] ReadyMix[™] PCR Reaction Mix (Sigma-Aldrich Company Ltd., Dorset, UK), 1 µl 10 pmol/µl forward and reverse primers, 2 µl bacterial DNA and 21 µl PCR-grade water. The primers used for this reaction are given in Table 2.1.

Primer	Sequence
8f (F-primer)	3'-CACGGATCCAGAGTTTGATYMTGGCTCAG-5'
1510r (R-primer)	3'-GTGAAGCTTACGGYTACCTTGTTACGACTT-5'

Table 2.1 – Universal primers used in the PCR amplification of the 16S gene

PCR amplification of the 16S gene was carried out on a Techne TC-312 thermo cycler (Bibby Scientific Limited, Staffordshire, UK). After an activation step of 3 min at 94 °C, PCR was carried out for 30 cycles under the following conditions: denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 68 °C for 30 s. After the 30 cycles, a final extension step was carried out at 68 °C for 7 min followed by termination by holding at 4 °C.

Copies of the target gene produced by the PCR reaction were purified using the QIAquick[®] PCR Purification Kit (Qiagen, Crawley, UK). All solutions were made up to the manufacturer's specifications and all consumables were provided with the kit. 5 volumes of Buffer PB were added to 1 volume of the PCR sample and the colour of the pH indicator I was checked to be yellow (indicates a pH of ≤ 7.5). The sample was added to a QIAquick spin column and centrifuged at 17,900 x g for 45 s. The flow through was discarded and 750 µl Buffer PE was added to the column and centrifuged at 17,900 x g for 45 s. The flow through was discarded and the column was centrifuged 17,900 x g for 1 min. The QIAquick

column was added to a clean 1.5 ml microcentrifuge tube and 30 µl Buffer EB was added to the middle of the QIAquick membrane. The column was left to stand for 1 min and subsequently centrifuged at 17, 900 x g for 1 min. The purified PCR sample was stored at – 80 °C until required.

To test that the sample contained the target gene, gel electrophoresis was utilised. A 1 % agarose/tris borate EDTA (TBE) gel was used (2 g agarose in 200 ml of 0.5x TBE) with the addition of 20 µl SYBR[®] safe DNA gel stain (Invitrogen[™] Life Technologies Ltd., Paisley, UK) to help visualise the bands of DNA. 5 µl of the purified PCR product was, along with 1 µl of blue/orange loading dye (6x) (Promega UK, Southampton, UK), loaded on to the gel. 1 kbp and 50 bp DNA step ladders (Figure 2.4) were also loaded on to the gel (6 µl and 2 µl respectively, with 8 µl PCR-grade water and 2 µl blue/orange loading dye added to the 50 bp ladder). The gel was run at 125 V and 200 mA for approximately 4 h, until the orange dye had reached the end of the gel. The resultant gel was visualised using a BioDoc-It Imaging System with an M20 (20x20cm filter) transilluminator (Ultra-Violet Products Ltd, Cambridge, UK) and the DNA band gained for PCR product sample was compared to the DNA ladders to determine the length of the DNA strands.

Once the gene was confirmed to be present, the sample was sent, along with the 8f and 1510r primers to Eurofins MWG Operon (Ebersberg, Germany) for custom DNA sequencing. The gene was sequenced forwards the utilising 8f primer and backwards using the 1510r primer. The two sequencing results were pieced together to give the whole gene sequence. The whole gene sequence was subjected to a BLASTn search of the ‘Nucleotide collection (nr/nt)’ database, with the ‘megablast’ option chosen. The results were sorted by ‘% match’ to give the best matches.

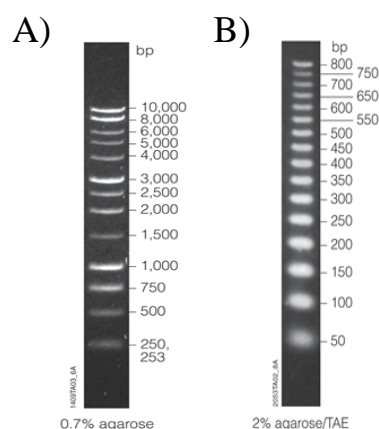


Figure 2.4 – DNA step ladders used in the gel electrophoresis of the PCR product sample. 1 kbp (A)) and 50 bp (B)) DNA step ladders were used to determine the length of the DNA strands in the PCR product sample.

2.10.6 cDNA production by reverse transcription (RT)-PCR

RNA isolated from epithelial cells was converted, by reverse transcription (RT) reaction, to cDNA for use in quantitative real-time (qRT)-PCR using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). 2 µg template RNA was added to 8 µl 5x iScript reaction mix and 2 µl iScript reverse transcriptase and the volume was made up to 40 µl with nuclease-free water. The complete reaction mix was incubated in a Techne TC-312 Thermocycler, using the following program: 5 min at 25 °C, 30 min at 42 °C, 5 min 85 °C and the reaction mix was held at 4 °C.

2.10.7 Relative quantification real-time (qRT)-PCR

Relative quantification real-time (qRT)-PCR was performed to investigate the relative fold change in gene mRNA expression in response to cell challenges, when compared to control conditions. qRT-PCR was performed on the carousel-based system of the Lightcycler® 2 machine (Roche Diagnostics Ltd., West Sussex, UK) and utilising custom PCR assays from the Human Universal ProbeLibrary set coupled with the gene-specific primers (Table 2.2).

Gene	Pubmed Accession No.	Primer Sequence		Universal Probe No.
β -actin	NM_001101	F – ccaaccgcgagaagatga	R – tccatcacgatgccagtg	#64
GAPDH	NM_002046	F – gctctctgctctctctgttc	R – acgaccaaatccgttgactc	#60
IL-8	NM_000584	F – agacagcagagcacacaagc	R – aggaaggctgccaagagag	#72
IL-10	NM_000572	F – tgccttcagcagagtgaaga	R – gcttggaaccaggtaa	# 65
TLR-1	NM_003263	F – aaacaacattgaaacaacttgaa	R – cacgttgaaattgagaaatacca	# 65
TLR-2	NM_003264	F – ctctcggtgtcggaatgtc	R – aggatcagcaggaacagagc	#56
TLR-4	U88880	F – gaaggttcccagaaaagaatgtt	R – cctgattgtccttttctgaatg	# 75
TLR-5	NM_003268	F – ctccacagtcaccaaacag	R – cctgtgtattgatgggcaaa	# 72
TLR-9	NM_017442	F – tgtgaagcatccttcctgta	R – gagagacagcgggtgcag	#56
CD14	AB446505	F – ggaagacttatcgaccatggag	R – acaaggttctggcgtggt	# 74
NOD1	AF113925	F – tcagggacagagtccctggtt	R – acccctcaggaaatgattga	# 79

Table 2.2 – Primers and Universal probes used in qRT-PCR. Gene-specific primer pairs were linked to a Universal ProbeLibrary probe by the online ProbeFinder software. F- indicates forward primer and R- indicates reverse primer.

The Universal ProbeLibrary consists of a set of 165 short (8-9 nucleotides) hydrolysis probes which are labelled at the 5' end with a reporter dye, fluorescein (FAM), and at the 3' end with a dark quencher dye. Custom-designed PCR assays are produced using the web-based ProbeFinder Software (<http://qpcr.probefinder.com/organism.jsp>) which optimally combines an appropriate Universal ProbeLibrary probe with a gene-specific primer pair. Where possible, the PCR amplicon selected by the software will span an exon-exon junction, reducing the risk of false positive results from genomic DNA contamination.

qRT-PCR reactions were carried out in a final volume of 20 μ l with the following reaction mix: 0.5 μ l 20 μ M forward and reverse primers, 0.5 μ l Universal ProbeLibrary probe, 4 μ l of 5x Lightcycler® Taqman® Master mix, 13 μ l PCR-grade water and 1.5 μ l cDNA sample. The PCR program used was an activation step at 95 °C for 10 min followed by 45 cycles of amplification at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s, and finally a cooling step at 40 °C for 30 s. When hydrolysis probes are intact, the quencher blocks the

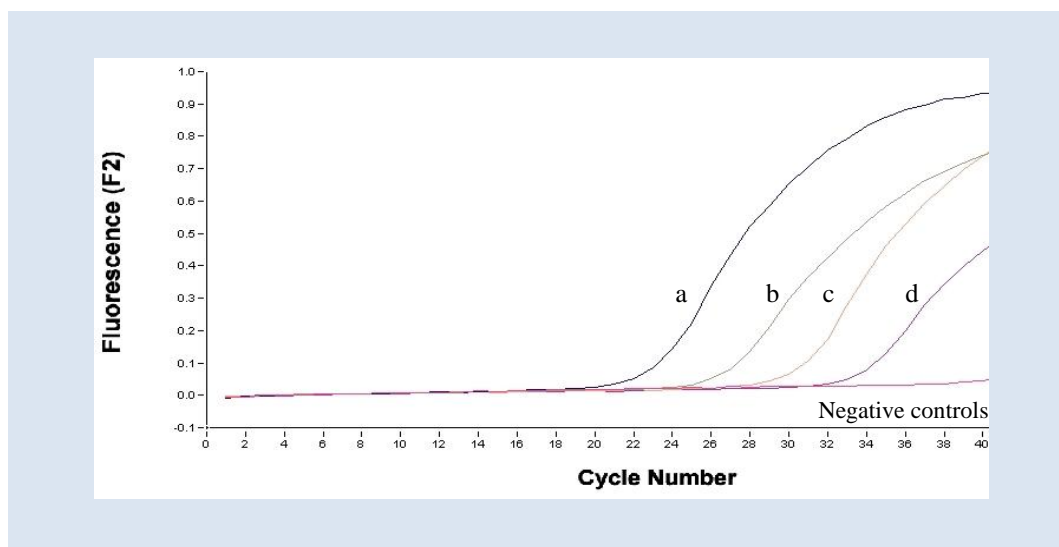


Figure 2.5 – Typical amplification curves of qRT-PCR. Serial dilutions of cDNA were subjected to qRT-PCR and the characteristic sigmoidal curves were evident. The amount of cDNA was a) 1x, b) 0.1x, c) 0.01x and d) 0.001x. This was reflected in the *Ct* values as a) gave a *Ct* value of 22.82, b) was 26.37, c) was 30.26 and d) was 33.39. The negative controls contained no cDNA and no reaction mix (just mRNA and water), respectively.

fluorescence of the reporter dye, however, when the Taq polymerase cleaves the probe, the dyes separate, allowing the reporter to fluoresce. Fluorescence was measured at 530 nm. The Lightcycler[®] 1.5 software was used to calculate the threshold cycle (*Ct*) value (the point at which the fluorescence exceeds the background fluorescence) (Figure 2.6) and the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) was used to analyse the fold change in mRNA expression in comparison with control conditions. In this method of analysis, genes of interest were normalised to the housekeeping genes (in this case, β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) to allow comparison between control and challenge conditions. Results were expressed as fold change in mRNA expression.

2.10.8 Validating PCR primers

PCR primer pairs, and indeed PCR reaction mixtures as a whole, underwent efficiency tests before use to confirm they fell within the acceptable limits of PCR efficiency (90-110 %). Briefly, the template cDNA sample was serially diluted and each concentration was subjected to qRT- PCR (Section 2.10.7). The C_t value was plotted against the \log_{10} of the dilution (e.g. for a 10x dilution, the $\log_{10}0.1$ was taken) and a linear trend line was fit to the resultant graph (Figure 2.7). The efficiency of the PCR reaction was calculated utilising the slope of the trend line and the following equation:

$$\text{Efficiency of PCR Reaction} = 10^{(-1/\text{slope})} \quad (\text{Equation 2.3})$$

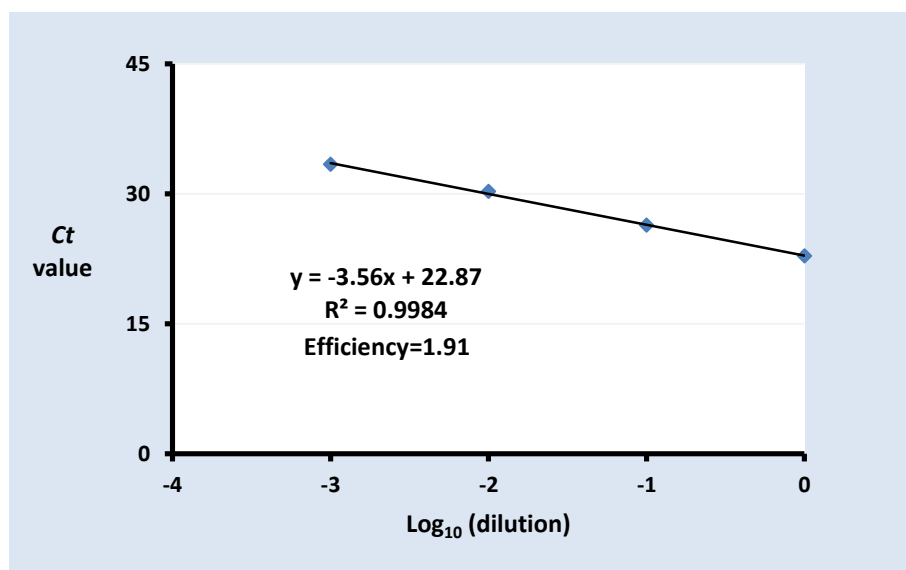


Figure 2.6 – Primer efficiency assessment. cDNA samples were serially diluted (in this case 1, 0.1, 0.01 and 0.001x were used) and subjected to qRT-PCR. The resultant C_t values were plotted against the \log_{10} of the dilution. Results show the efficiency of the IL-8 primers (see Table 2.2).

2.11 Statistical analysis

Results were all expressed as mean \pm standard error of the mean (SEM) for the specified number of experimental repeats (n). Statistical significance was resolved using unpaired Student's t-test or ANOVA analysis (used where multiple comparisons are required within an experiment) and defined as a p -value ≤ 0.05 and/or p -value ≤ 0.01 . All statistical analyses were undertaken using PASW[®] Statistics 17 (IBM, Armonk, NY, USA). Statistical difference is indicated by * or **, where $p \leq 0.05$ or 0.01 , respectively.

Chapter 3

Characterisation of the HT29-19A and Caco-2 intestinal epithelial cell models

3.1 Introduction

Immortalised *in vitro* cell lines offer a very robust and easily reproducible alternative to animal models (*in vivo*) or primary cell explants (*ex vivo*). Caco-2 cells and the 19A clonal derivative of the HT29 cell line offer a highly simplified model of the *in vivo* intestinal epithelial layer, as both originate from human colonic adenocarcinomas. Nevertheless, both cell lines are phenotypically distinct (Warhurst *et al.*, 1998) and have previously been used in a wide variety of different physiological investigations (LeFerrec *et al.*, 2001). For example, the Caco-2 cell line is often used in the pharmaceutical industry for drug development (Bailey *et al.*, 1996) and intestinal absorption modelling (Artursson *et al.*, 2001; Sambuy *et al.*, 2005; Hilgers *et al.*, 1990), whereas HT29-19A cells have previously been used in studies of protein transport (Terpend *et al.*, 1998), epithelial differentiation (Cohen *et al.*, 1999) and chemokine secretion (Warhurst *et al.*, 1998). Additionally, both cell lines have also been used to model bacterial interactions with the intestinal epithelium, with Caco-2 cells extensively used in studies of bacterial adherence (Coconnier *et al.*, 1993; Coconnier *et al.*, 1997; Tuomola and Salminen, 1998) and translocation (Cruz *et al.*, 1994; Clark *et al.*, 2003; Clark *et al.*, 2005) and HT29-19A cells used in inflammation stimulatory studies (Lammers *et al.*, 2002; Bannon *et al.*, 2009).

The two intestinal epithelial cell lines HT29-19A and Caco-2 were characterised, in terms of their pattern recognition receptor expression, expression of IL-8 and IL-10 and their sensitivity to commercially available bacterial TLR agonists. Additionally, disruption of the epithelial barrier function and stimulation of the proinflammatory response by factors released by the enteric pathogen *Clostridium difficile* were investigated.

3.2 A quantitative comparison of mRNA expression of Toll-like receptors (TLRs) involved in bacterial antigen recognition in intestinal epithelial cell lines (IECs)

A quantitative analysis of the expression of PRRs, specifically TLRs, was undertaken. TLR expression in HT29-19A and Caco-2 cells has previously been qualitatively investigated by reverse transcription (RT)-PCR by Bannon (2008), who reported constitutive expression of TLRs 1-10 in both cell lines. However, analysis undertaken in this study was limited to TLR-1, TLR-2, TLR-4, TLR-5, and TLR-9, and was semi-quantitative in nature.

This initial study demonstrated that both cell lines express a range of TLRs required for the recognition and response to a wide range of bacterial antigens, with TLR-1, TLR-2, TLR-4, TLR-5, and TLR-9 all exhibiting a detectable signal (Table 3.1). These show the phenotypic differences between the two cell lines, as Caco-2 cells had a significantly higher expression of TLR-2 mRNA than HT29-19A cells (~700-fold; $p \leq 0.01$; Figure 3.1). In contrast, HT29-19A cells exhibited much greater expression of TLR-4 mRNA than the Caco-2 cell line (~340-fold; $p \leq 0.01$; Figure 3.1). TLRs-1, -5 and -9 were all constitutively expressed at similar levels in the two cell lines (Figure 3.1). However, it is worth noting that, upon observation of the raw *Ct*-values (Table 3.1), TLR-5 mRNA appeared to be present in moderately increased levels in both cell lines, when compared to the mRNA expression of both TLR-1 and TLR-9 (*NB*. *Ct*-values are logarithmic).

Gene	Ct value		p-value	
	HT29-19A	Caco-2	≤ 0.05	≤ 0.01
Actin	16.06 ± 0.24	15.44 ± 0.23	-	-
GAPDH	18.83 ± 0.12	18.56 ± 0.17	-	-
TLR-1	32.32 ± 0.16	32.75 ± 0.25	-	-
TLR-2	36.67 ± 0.29	26.53 ± 0.12	+	+
TLR-4	28.02 ± 0.26	35.73 ± 0.29	+	+
TLR-5	28.50 ± 0.33	28.14 ± 0.22	-	-
TLR-9	35.15 ± 0.38	36.12 ± 0.65	-	-

Table 3.1- Raw Ct values of TLR mRNAs in IECs. The raw threshold (*Ct*) values were taken from qRT-PCR and cell lines were compared for statistical significance. Results are mean ± SEM, n = 4.

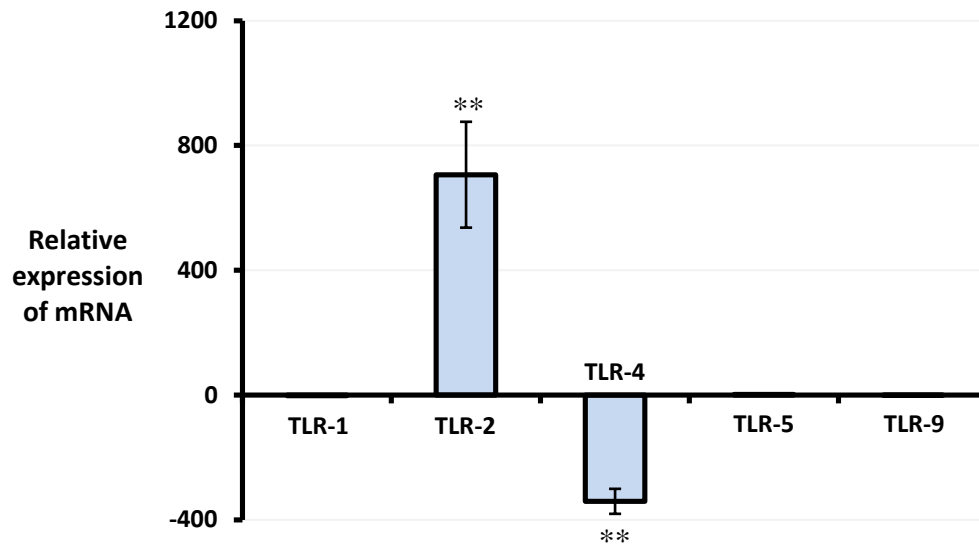


Figure 3.1 –Relative expression of TLR mRNA in Caco-2 vs. HT29-19A cells. Results were normalised to housekeeping genes, β -actin and GAPDH, and expressed as a fold difference in Caco-2 cells when compared to HT29-19A cell line. Results are mean \pm SEM, n = 4.

3.3 Constitutive expression of IL-8 and IL-10 in IECs

To examine whether the HT29-19A and Caco-2 cell lines spontaneously expressed IL-8 and IL-10, ELISA and qRT-PCR analyses were employed to measure the levels of the cytokines at the protein and mRNA levels, respectively.

The cells lines exhibited different profiles of IL-8 release, over 24 h. Both cell lines released detectable levels of IL-8 at the 1 h time point, however the release in HT29-19A cells was ~12-fold higher than in Caco-2 cells. Subsequently, HT29-19A cells released IL-8 in a time-dependent manner, whereas Caco-2 cells exhibited little change from 1 to 12 h, and only showed a small increase between the 12 and 24 h time points. At the 24 h, the IL-8 release by HT29-19A cells was ~64-fold higher than Caco-2 cells (Figure 3.2). In concordance with these data, HT29-19A cells were seen to express a significantly higher level of IL-8 mRNA than Caco-2 cells (~73-fold; Figure 3.3).

Neither cell line released any detectable amounts of IL-10 (data not shown). Furthermore, HT29-19A cells did not express detectable levels of IL-10 mRNA within the 45 cycles of amplification during qRT-PCR, however, IL-10 mRNA expression was detected in Caco-2 cells. For purposes of quantitative comparison, the *Ct*-value for HT29-19A cells was taken as 45, thus allowing a minimum fold difference to be calculated. IL-10 mRNA expression was demonstrated to be >227-fold greater in Caco-2 cells when compared to HT29-19A cells (Figure 3.3).

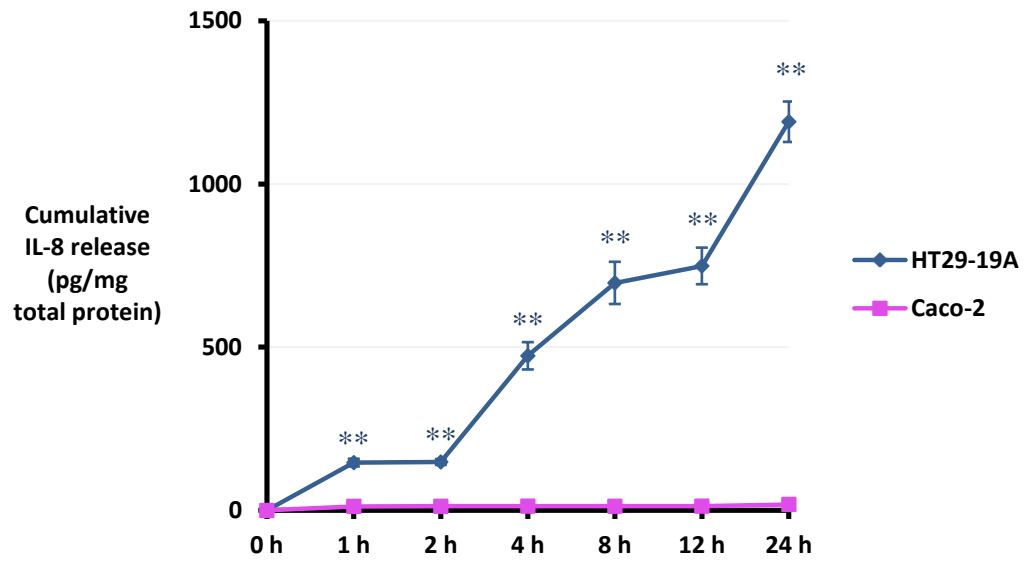


Figure 3.2 – Basal level release of IL-8 is significantly higher in HT29-19A cells than Caco-2 cells. Results are normalised to protein concentration from cell lysates and expressed as mean \pm SEM, n = 4.

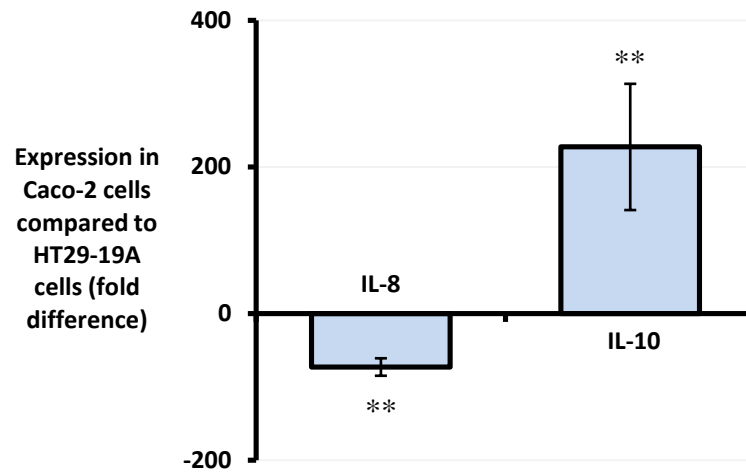


Figure 3.3 – Quantitative comparison of chemokine mRNA expression in IECs. Results were normalised to the actin and GAPDH housekeeping genes. Results are expressed as a fold difference in the Caco-2 when compared to HT29-19A cell line and are mean \pm SEM, n = 4.

3.4 Interaction of TLR-agonists with IECs

Cells were challenged with commercially available bacterial antigens to explore the expression of IL-8 under stimulatory conditions. HT29-19A cells demonstrated a much higher capacity for IL-8 production than Caco-2 cells, with significantly more IL-8 released in response to maximal concentrations of peptidoglycan (~42-fold), lipopolysaccharide (~61-fold), flagellin (~27-fold) and CpG DNA (~61-fold), respectively (Figure 3.4). Additionally, the relative sensitivities were varied between the two cell lines. Caco-2 cells were significantly (100-fold; $p \leq 0.01$) more sensitive to peptidoglycan than HT29-19A cells, with a threshold level of 5 $\mu\text{g/ml}$, compared to 500 $\mu\text{g/ml}$ (Figure 3.4a). Conversely, Caco-2 cells were unresponsive to the concentration range of LPS tested, whereas HT29-19A cells showed a relatively low threshold level of 10 ng/ml , thus demonstrating >100-fold reactivity (Figure 3.4b). In response to flagellin and CpG DNA, both cell lines showed similar thresholds of 1 ng/ml (Figure 3.4c) and 10 $\mu\text{g/ml}$ (Figure 3.4d), respectively. These results suggest that, due to their variations in TLR expression patterns (see Section 3.2), the two cell lines exhibit different sensitivities to the range of TLR agonists.

To investigate if the potentiation of IL-8 protein secretion, in response to the bacterial antigens, was paralleled by the mediation of transcriptional expression, IL-8 mRNA levels were measured by qRT-PCR. In the HT29-19A cell line, cells incubated with peptidoglycan (500 $\mu\text{g/ml}$), lipopolysaccharide (1000 ng/ml), flagellin (100 ng/ml) or CpG DNA (50 $\mu\text{g/ml}$) exhibited significantly ($p \leq 0.05$) more IL-8 mRNA expression than unstimulated controls (Figure 3.5a), when normalised to the housekeeping genes. Caco-2 cells showed a similar pattern, with IL-8 mRNA significantly up-regulated in all challenges (Figure 3.5b).

Changes in expression of TLRs in response to bacterial antigens were also investigated. In HT29-19A cells, incubation with peptidoglycan, lipopolysaccharide and flagellin all resulted in significant ($p \leq 0.05$) up-regulation of their cognate receptors (Figure

3.5). Additionally, mRNA expression of CD14 appeared to be up-regulated (14.11 ± 6.49 -fold) in response to lipopolysaccharide; however, the variability in expression meant that the result was not significantly different from the expression level in control cells. Conversely, in reaction to CpG DNA, a considerable ($p \leq 0.01$) down-regulation of TLR-9 was observed, with no detectable levels present in the challenged cells.

In Caco-2 cells, significant ($p \leq 0.05$) augmentations of TLR-1 and TLR-2, TLR-4, TLR-5 and TLR-9 mRNAs were demonstrated in response to peptidoglycan, lipopolysaccharide, flagellin and CpG DNA, respectively. In contrast to this, expression of CD14 mRNA was significantly ($p \leq 0.01$) diminished, in response to lipopolysaccharide.

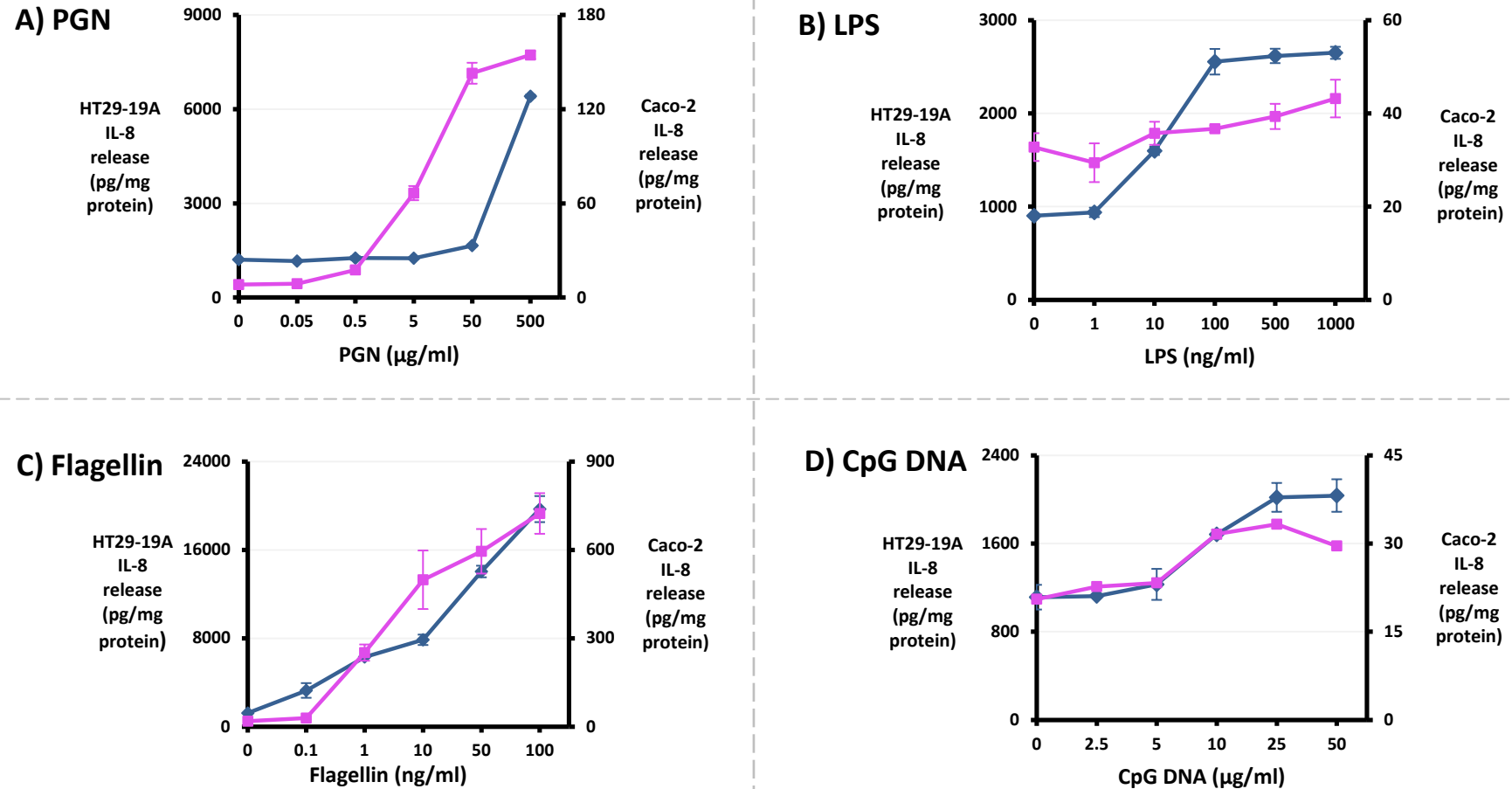


Figure 3.4 – Known TLR agonists elicit IL-8 release from IECs in a dose-dependent manner. Epithelial cells were challenged with either peptidoglycan (PGN) from *B. subtilis* (A), lipopolysaccharide (LPS) from *E. coli* O111:B4 (B), flagellin from *S. typhimurium* strain 14028 (C) or deoxyribonucleic acid sodium salt (CpG DNA) from *Escherichia coli* strain B (D), for 24 h. IL-8 release was measured and is expressed as mean \pm SEM, n = 3. $\text{—}\blacklozenge\text{—}$ = HT29-19A cells, $\text{—}\blacksquare\text{—}$ = Caco-2 cells.

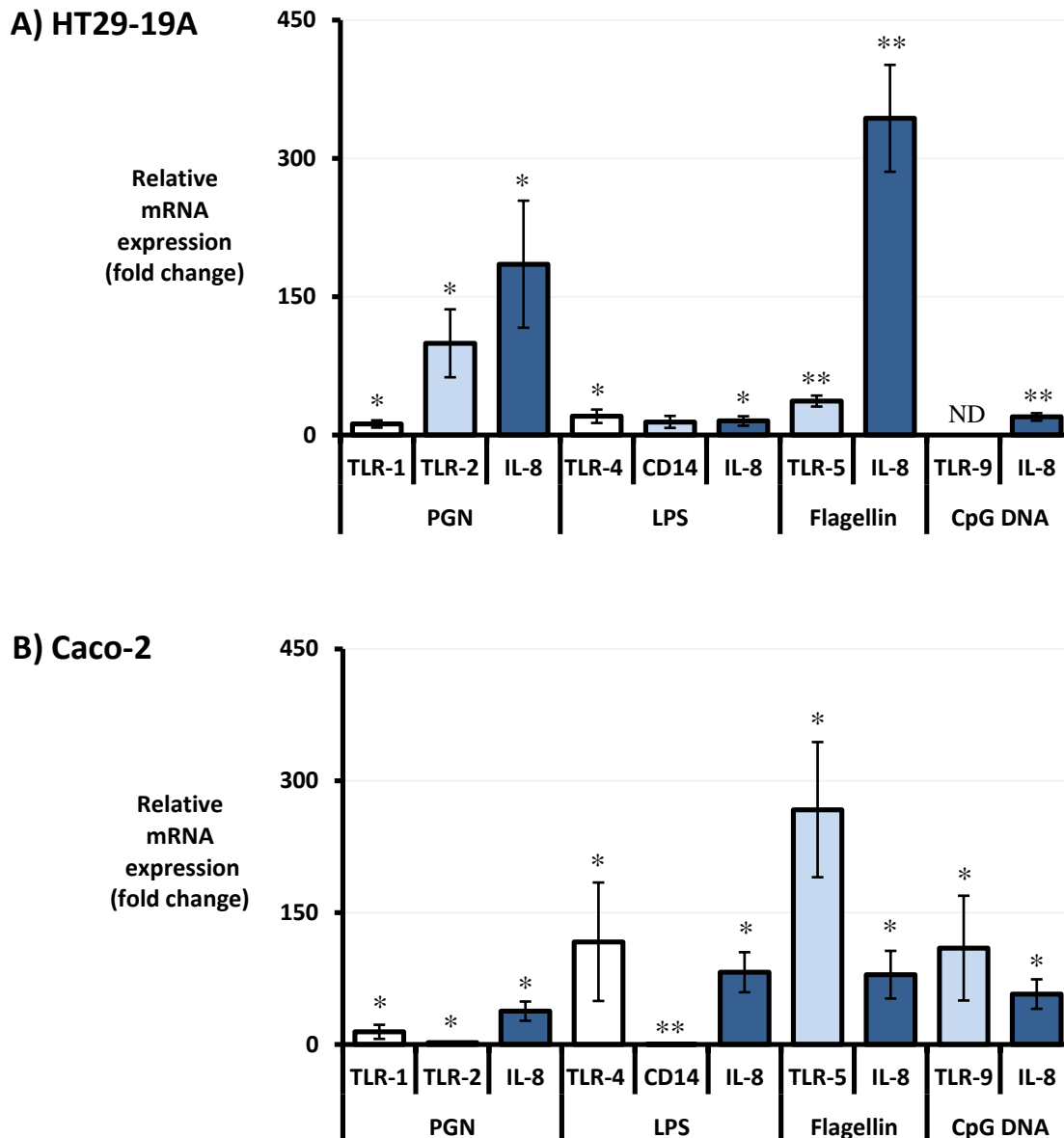


Figure 3.5 – TLR agonists up-regulate IL-8 and cognate TLR mRNA expression in IECs. HT29-19A (A) and Caco-2 (B) cells, were challenged with either 500 µg/ml peptidoglycan (PGN), 1000 ng/ml lipopolysaccharide (LPS), 100 ng/ml of flagellin or 50 µg/ml of deoxyribonucleic acid sodium salt (CpG DNA) for 24 h. Results are normalised to the actin and GAPDH housekeeping gene and expressed as a fold difference in Caco-2 cells, compared to HT29-19A cells. Results are mean ± SEM, n = 3. ND = not detected.1;;

3.5 IL-8 expression in IECs, in response to factors released by *C. difficile*

Inflammatory effects of *C. difficile* on HT29-19A cells were dose-dependent, with HT29-19A cells challenged with a 1 in 10 dilution of *C. difficile* crude cfs demonstrating a significant increase in IL-8 release (~15-fold; $p \leq 0.01$), when compared to secretion by vehicle control (HB media)-challenged cells (19642 ± 1896.7 compared to 1322 ± 115.7 pg/mg total protein). The effects were also evident at the 1 in 100 and 1 in 1000 dilutions, but lost at a 1 in 10,000 dilution (Figure 3.6a). A similar 15-fold increase in IL-8 secretion, in response to 1 in 10 dilution of *C. difficile* cfs, was also observed in Caco-2 cells; however, more dilute challenge samples had no effect (Figure 3.6b).

To explore whether the protein level results were reflected at the transcriptional level, IL-8 mRNA expression, in response to a 1 in 10 dilution of *C. difficile* cfs, was measured by qRT-PCR. The results showed significant increases, of comparable magnitude, in IL-8 mRNA in both cell lines, with ~113- and ~98-fold increases, respectively (Figure 3.7).

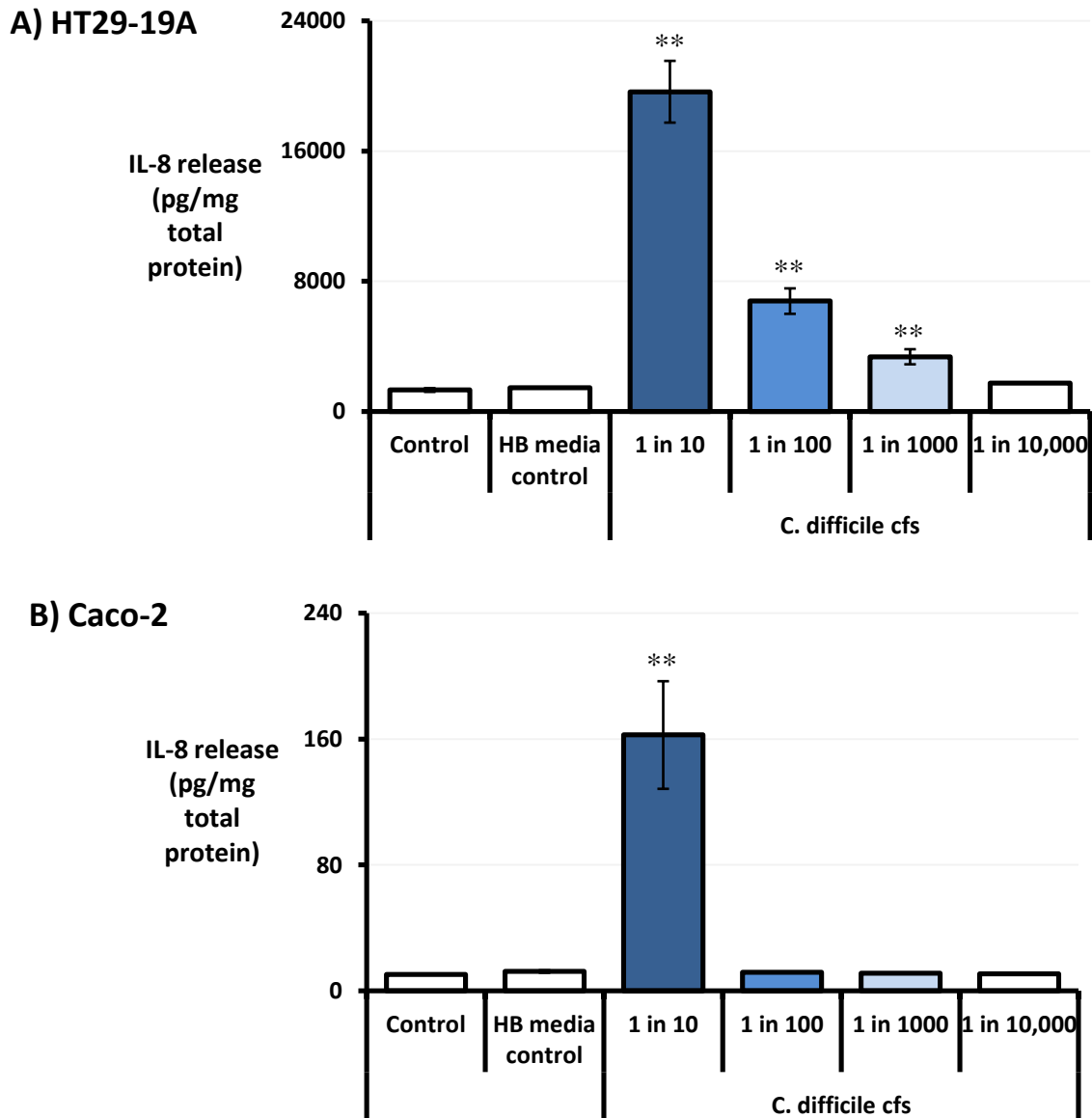


Figure 3.6 – *C. difficile* cell-free supernatant stimulates IL-8 release from IECs. HT29-19A (A) and Caco-2 (B) cells were challenged with 1 in 10 to 1 in 10,000, dilutions of *C. difficile* cell-free supernatant and 1 in 10 dilution of horse blood media (vehicle control) for 24 h. Supernatants were analysed for IL-8. Results are mean \pm SEM, n = 6. ** indicates significance from HB media control.

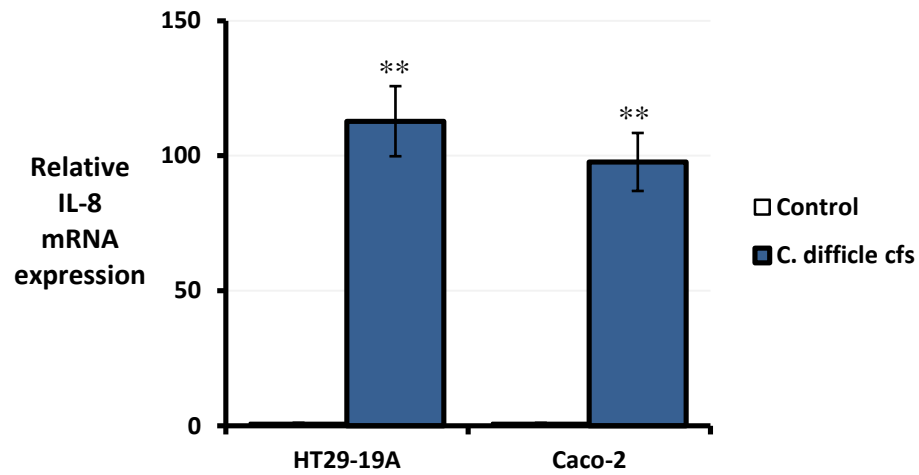


Figure 3.7 – *C. difficile* cell-free supernatant elicits significant up-regulation of IL-8 mRNA expression in IECs. Cells were challenged with a 1 in 10 dilution of *C. difficile* cell free supernatant, for 24 h, and IL-8 mRNA was analysed. Results are mean \pm SEM, n=3.

3.6 High concentrations of *C. difficile* cell-free supernatant irreversibly disrupt the barrier function of Caco-2 monolayers

HT29-19A cell layers were unable to polarise sufficiently to form dome structures (Figure 3.8), however, Caco-2 cells readily polarised to the extent that a considerable number of dome structures were maintained from day 7 to day 18 (Figure 3.8). Subsequent to day 18, the dome count halved, with the number dropping from 104.9 ± 9.4 on day 18 to 51.9 ± 9.6 on day 19 (Figure 3.2). The decline in dome number could, in all probability, be attributed to a natural weakening of the tight junctions or death of the cells, due to their age. These data indicate that studies to be undertaken on barrier function in Caco-2 cells should be done so on monolayers aged 7-18 days.

7-day old monolayers of Caco-2 cells were challenged with varying concentrations (neat to 1 in 100,000) of *C. difficile* cell-free supernatant (cfs), and its vehicle control, horse blood (HB) media, for 24 h. All domes were lost immediately following the challenge (Figure 3.9). In the subsequent 96 h, cells challenged with neat cfs showed no recovery of domes. Additionally, cell monolayers challenged with 1 in 10 and 1 in 100 dilutions of cfs showed very few domes, with numbers significantly ($p \leq 0.01$) lower than control cells. However, after 48 h, cells challenged with greater dilutions of cfs (1 in 1000, 1 in 10,000 and 1 in 100,000) all showed a return to dome numbers comparable to control levels.

These results showed that high concentrations of *C. difficile* cfs irreversibly eradicated domes in Caco-2 cells. Conversely, the acute disruptive effect of lower concentrations of cfs diminished after 48 h and a temporary, but significant, increase in dome numbers was observed before they returned to control level. The suitability of *C. difficile* cell-free supernatant in barrier function studies in Caco-2 cells was established.

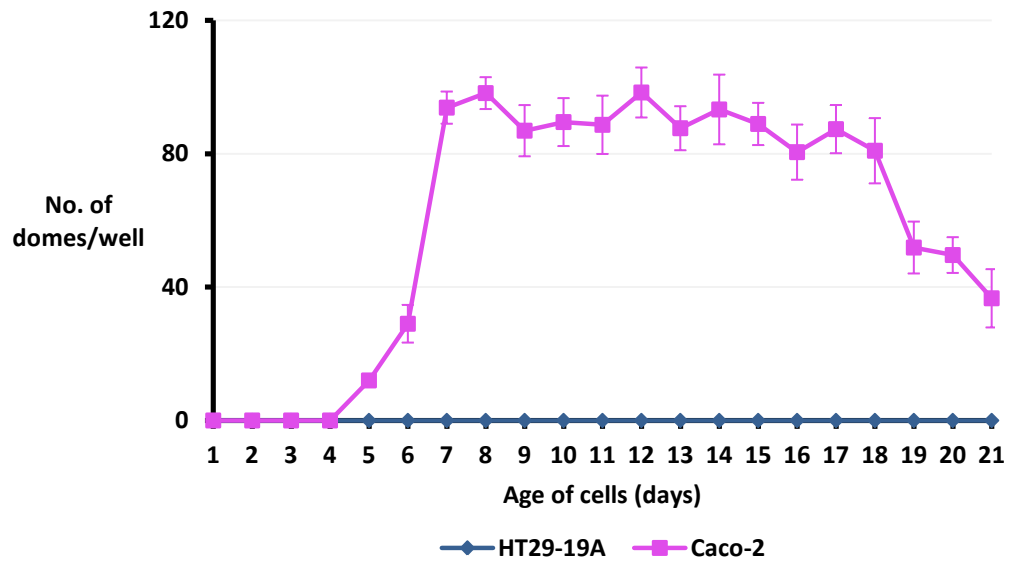


Figure 3.8 – Caco-2 monolayers polarise sufficiently enough to form domes. Results are expressed as number of domes per well and are mean \pm SEM, n = 10–12.

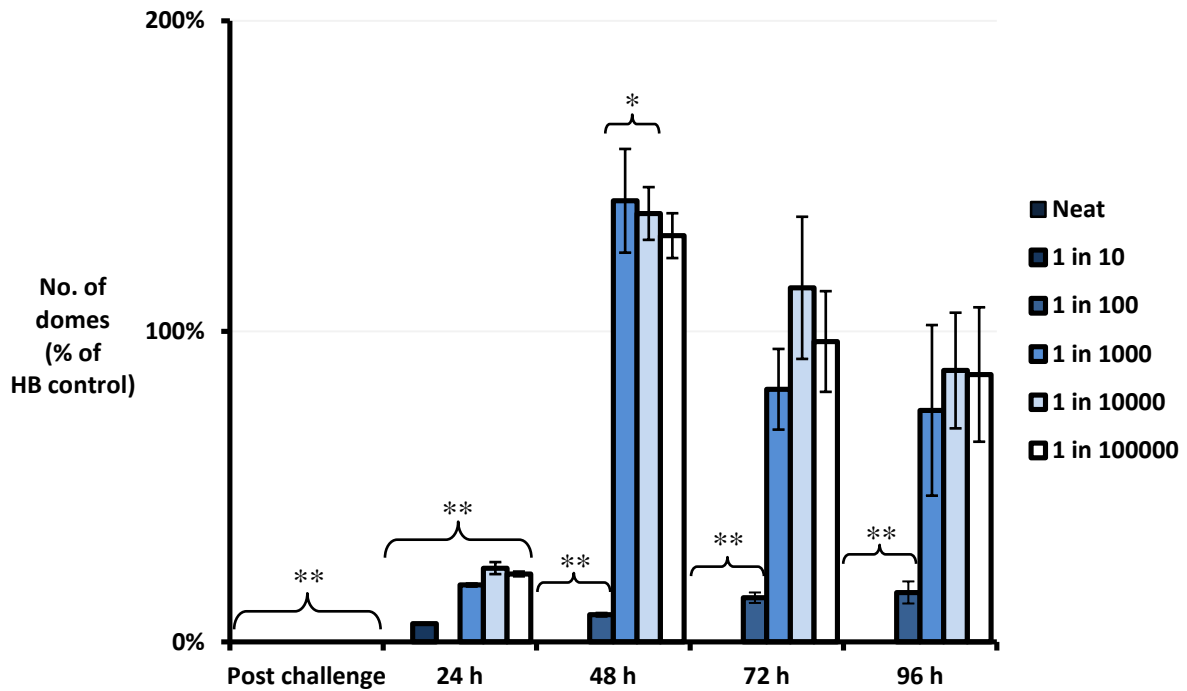


Figure 3.9 – *C. difficile* cell-free supernatant significantly diminishes barrier integrity of Caco-2 monolayers. Caco-2 cells were challenged with neat to 1 in 100,000 dilutions of horse blood (HB) media and *C. difficile* cell-free supernatant samples for 24 h. Results are expressed as a % of number of domes observed in the HB media vehicle control. Results are mean \pm SEM, n=6. ** indicates significance from 100 %.

3.7 Summary

This chapter explored differences between the two intestinal epithelial cell lines HT29-19A and Caco-2 with the overall aim of selecting the most appropriate for subsequent experimentation in this investigation.

A quantitative comparison of the TLRs, undertaken by qRT-PCR, showed significant differences in TLR-2 and TLR-4 mRNA between the two cell lines, with HT29-19A cells exhibiting significantly higher expression of TLR-4, but considerably less expression of TLR-2, than Caco-2 cells. Conversely, the two cell lines exhibited comparable transcriptional expression of TLR-1, TLR-5 and TLR-9. Proinflammatory and anti-inflammatory cytokines (IL-8 and IL-10, respectively) were also investigated, with HT29-19A cells demonstrating a larger capacity, at both the transcriptional and translational levels, for IL-8 expression than the Caco-2 cell lines, but, in contrast to Caco-2 cells, they did not exhibit expression of IL-10 mRNA.

Having established constitutive expression of TLRs and IL-8, modulatory effects following cell stimulation were explored. The HT29-19A cell line showed significantly higher maximal release of IL-8 in response to all stimulants tested compared to Caco-2 cells. Nevertheless, the two cell lines were shown to exhibit similar sensitivity thresholds to both flagellin and CpG DNA. Conversely, owing to their TLR-2 and TLR-4 mRNA profile, HT29-19A cells were significantly more reactive to LPS, but considerably less sensitive to peptidoglycan, than Caco-2 cells. Additionally, the bacterial antigens tested were shown to up-regulate the mRNA expression of both IL-8 and their cognate TLR receptors in both cell lines. *C. difficile* cell-free supernatant was also shown to elicit the expression and release of IL-8 from both cell lines.

Finally, the ability of the two cell lines to polarise, forming dome structures, was investigated, and subsequently showed that Caco-2 cells were able to form and maintain a

significant number for ~14 days post-confluence. In contrast, HT29-19A cells were unable to form domes at all. Dome loss in response to *C. difficile* cell-free supernatant was utilised to explore its disruptive effects on integrity of Caco-2 monolayers.

Taken altogether, the results of this preliminary characterisation of the HT29-19A and Caco-2 intestinal epithelial cell lines demonstrated that the two cell lines are phenotypically divergent, but highly complementary in modelling the interaction of bacterial products with the intestinal epithelium. Therefore, both cell lines were utilised in the study of enteric-associated bacterial products considered in subsequent chapters.

Chapter 4

***E. coli* C25: investigating the inflammatory potential of a Gram-negative
commensal**

4.1 Introduction

Although the intestinal microflora is, potentially, a vast source of a wide range of immunostimulatory material (see Section 1.5), there has, to date, been little work undertaken with commensal-derived extracellular products. Most characterisation studies utilise commercially available bacterial antigens (as were used in the previous chapter (see Section 3.4)), which are typically isolated from pathogenic bacteria. The inflammatory potential of antigens produced by the Gram-negative commensal bacterium, *Escherichia coli* C25, was tested.

E. coli are among the first bacteria to colonise the human neonatal intestine (Hooper, 2004) and offer an early defence against enteropathogens, such as *Salmonella typhimurium* (Hudault *et al.*, 2001). *E. coli* C25 was originally isolated from the faeces of a healthy individual in the mid-1950s (Freter and Hentges, 1956), and has long been considered a commensal bacterium, with early studies demonstrating its antagonistic activities against the growth the enteric pathogen, *Shigella flexneri* (Hentges and Freter, 1962; Freter, 1962). Moreover, C25 lacks the traditional virulence genes found in pathogenic strains of *E. coli*, such as extraintestinal pathogenic (ExPEC), enterohaemorrhagic (EHEC) and enteropathogenic (EPEC) (Zareie *et al.*, 2005; Macutkiewicz *et al.*, 2008), and is a poor recipient of plasmid transfer (Freter and Brickner, 1983), so is unlikely to acquire such genes from other bacteria. Despite this, studies utilising live C25 have demonstrated its ability to initiate a proinflammatory response in intestinal epithelial cell lines (Michalsky *et al.*, 1997; Zareie *et al.*, 2005), however, the immunostimulatory ability of the extracellular products from C25 have only been briefly considered previously (Bannon, 2008).

4.2 *E. coli* C25 cell-free supernatant stimulates a proinflammatory response in IECs

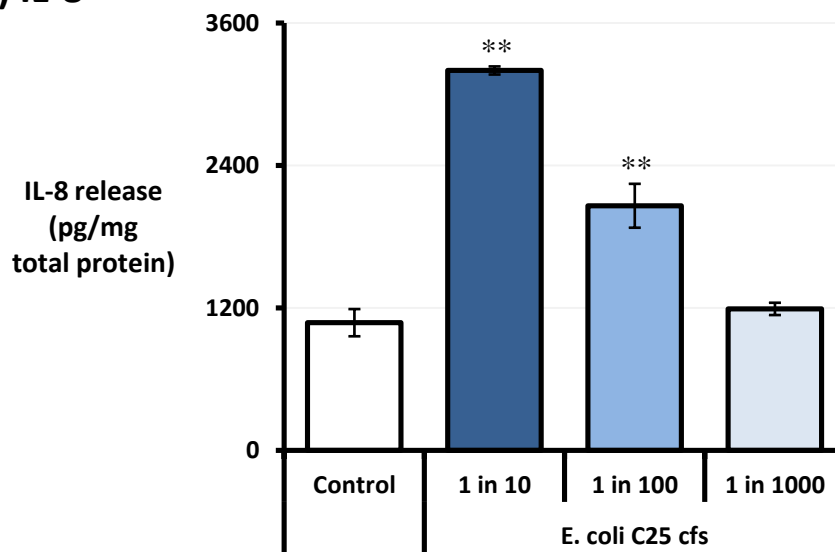
In response to a 1 in 10 dilution of *E. coli* C25 cell-free supernatant, HT29-19A cells exhibited a significant (~ 3 -fold; $p \leq 0.01$) increase in IL-8, from the basal level (3201 ± 34.1 compared to 1076 ± 114.6 pg/mg protein; Figure 4.1a), whereas Caco-2 cells showed a 1.5-fold increase, in comparison to the control (25.1 ± 0.6 vs. 16.7 ± 0.2 pg/mg protein; Figure 4.2a). A 1 in 100 dilution of the cell-free supernatant also induced a statistically significant ($p \leq 0.01$) IL-8 release in HT29-19A cells (2061.5 ± 184.9 pg/mg protein), but by the 1 in 1000 dilution, no increase was observed (Figure 4.1a). Conversely, Caco-2 cells, the control level of IL-8 was reached at the 1 in 100 dilution (Figure 4.2a). No detectable traces of IL-10 protein release were observed in either cell line (data not shown).

In parallel to IL-8 release, significant ($p \leq 0.01$) up-regulation of IL-8 mRNA was observed in both HT29-19A (~ 12 -fold; Figure 4.1b) and Caco-2 cells (~ 8 -fold; Figure 4.2b), in response to a 1 in 10 dilution of C25 cfs. Interestingly, both cell lines also demonstrated significant ($p \leq 0.01$) up-regulation of the key anti-inflammatory mediator IL-10, at the mRNA level, in response to the same dilution of C25 cfs. In contrast to unchallenged HT29-19A cells (which exhibited no detectable levels of IL-10 expression within the 45 amplification cycles of qRT-PCR), IL-10 mRNA was observed with a C_t value of 33.74 ± 0.47 , in cells challenged with C25 cfs (Figure 4.1c). Caco-2 cells showed a ~ 12 -fold increase in IL-10 mRNA, compared to the constitutive expression (Figure 4.2c).

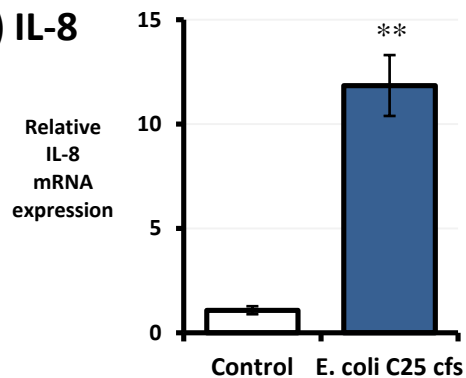
To investigate if the elicitation of increased IL-8 expression, in response to C25 cfs, was associated with changes in expression of TLR-1, TLR-2, TLR-4, TLR-5 and TLR-9, the fold change in mRNA expression of the receptors was quantified following cell challenges. A 1 in 10 dilution of C25 cfs provoked a significant ($p \leq 0.05$) up-regulation of TLR-1 mRNA in HT29-19A cells and Caco-2 cells (Table 4.1). Additionally, up-regulation in expression of TLR-4 and TLR-9 mRNA was also observed in both cell lines (Table 4.1). The expression of

TLR-2 and CD-14 did not deviate significantly from the transcriptional levels seen in unchallenged controls of both cell lines.

A) IL-8



B) IL-8



C) IL-10

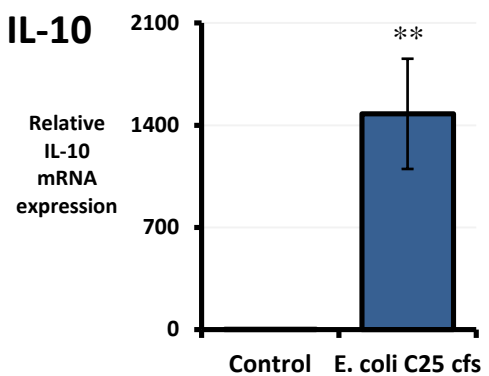


Figure 4.1 – *E. coli* C25 cell-free supernatant significantly stimulates IL-8 release in HT29-19A cells. HT29-19A cells were challenged with 1 in 10 to 1 in 1000 dilutions of *E. coli* C25 cell-free supernatant (cfs) for 24 h. Secretion of IL-8 (A) and transcriptional expression of IL-8 (B) and IL-10 (C), were measured. Results are mean \pm SEM, n=3-6.

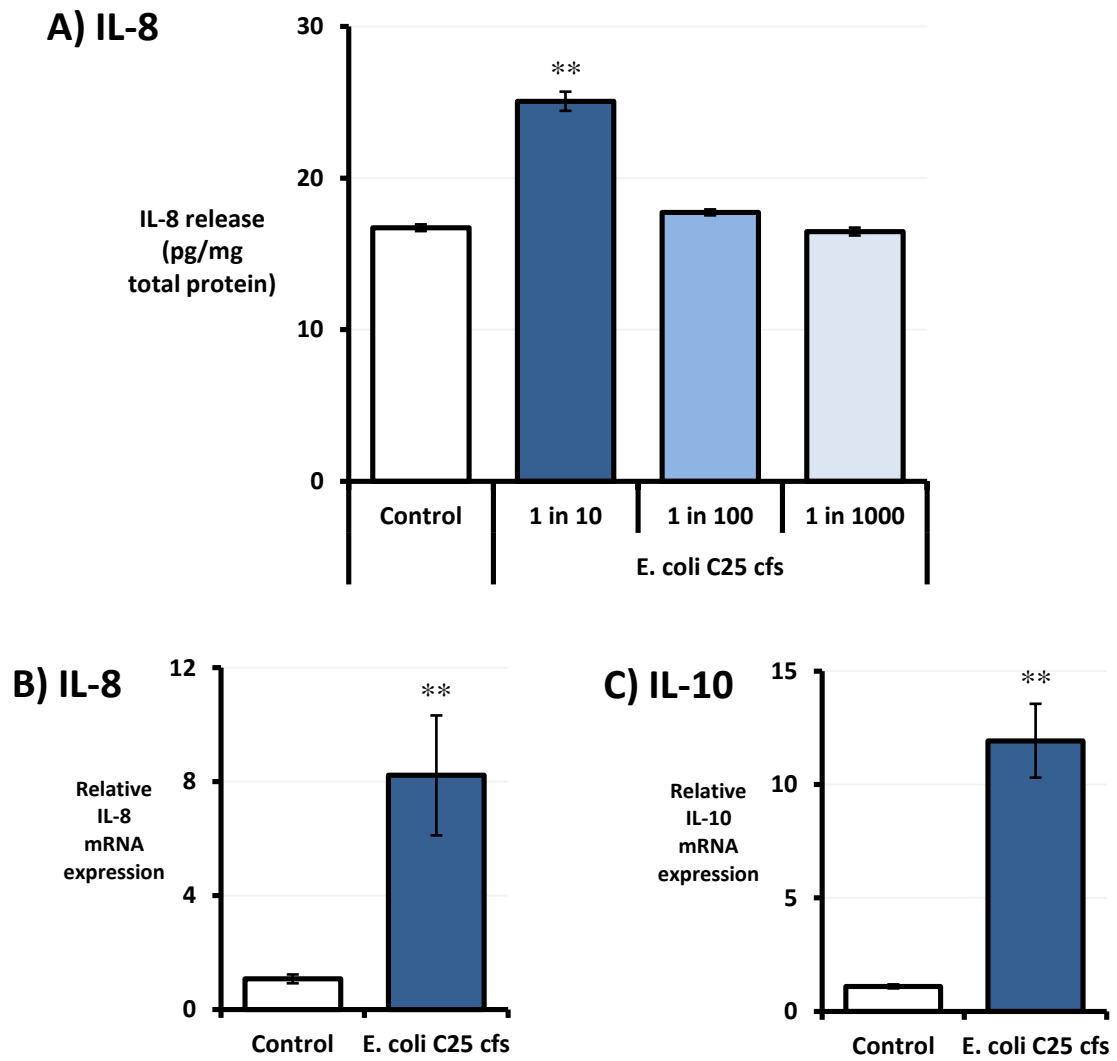


Figure 4.2 – *E. coli* C25 cell-free supernatant significantly potentiates IL-8 release in Caco-2 cells. Caco-2 cells were challenged with 1 in 10 to 1 in 1000 dilutions of *E. coli* C25 cell-free supernatant (cfs) for 24 h. Secretion of IL-8 (**A**) and transcriptional expression of IL-8 (**B**) and IL-10 (**C**), were measured. Results are mean \pm SEM, n=3-6.

Gene	Fold difference in mRNA	
	HT29-19A	Caco-2
TLR-1	1.93 ± 0.39*	2.76 ± 0.37**
TLR-2	1.79 ± 0.37	1.25 ± 0.20
TLR-4	2.79 ± 0.48**	3.21 ± 0.98*
TLR-9	11.22 ± 3.56**	22.55 ± 4.53**
CD14	0.75 ± 0.11	0.73 ± 0.19

Table 4.1 – *E. coli* C25 cell-free supernatant differentially modifies TLR expression in IECs. IECs were challenged with 1 in 10 dilutions of *E. coli* C25 cell-free supernatant and mRNA expression of TLRs was investigated. Results are mean ± SEM, n = 3.

4.3 *E. coli* C25 sonicates potentiate IL-8 expression in IECs

Ultrasonic disruption of the bacterial cell wall was used to increase the release of antigenic material, which would normally be released in very low quantities. Intestinal epithelial cells challenged with a 1 in 10 dilution of C25 sonicate were shown to elicit significantly ($p \leq 0.01$) more IL-8 than control cells. Challenged HT29-19A cells produced ~4-fold more IL-8 (4413.3 ± 828.4 compared to 1076.4 ± 114.6 pg/mg protein; Figure 4.3a), while challenged Caco-2 cells nearly doubled the IL-8 release (30.8 ± 0.3 compared to 16.7 ± 0.2 pg/mg protein; Figure 4.3b). In comparison to earlier data of IL-8 release in response to a 1 in 10 dilution of C25 cfs (Section 4.2), the IL-8 release in response to C25 sonicate was higher in both cell lines, but only statistically significant ($p \leq 0.01$) in Caco-2 cells (Figure 4.3b). A 113.6-fold (HT29-19A cells; Figure 4.3c) and 19.9-fold (Caco-2 cells; Figure 4.3d) increase of IL-8 mRNA also occurred.

Having established the proinflammatory potential of *E. coli* C25 sonicates, the modulatory effects of the lysed bacteria on TLR receptor expression in the two cell lines was investigated. A 1 in 10 dilution of C25 sonicate stimulated significant up-regulation of TLRs-2 and -4 mRNA in both the HT29-19A (~188- and ~44-fold, respectively; $p \leq 0.05$) and Caco-2 (~3.2- and ~28.7-fold, respectively; $p \leq 0.05$) cell lines (Table 4.2). TLR-1 mRNA expression was also significantly ($p \leq 0.01$) increased in Caco-2 cells, and appeared to be up-regulated in HT29-19A cells, however, this was not statistically significant due to the large variance in data (Table 4.2). In HT29-19A cells, CD14 mRNA expression in response to C25 sonicate did not significantly differ from the control expression, but, in Caco-2 cells, the expression was significantly ($p \leq 0.05$) diminished (Table 4.2). Challenge by C25 sonicates also caused significant down-regulation of TLR-9 mRNA in both cell lines, so much so that no detectable traces were present (Table 4.2).

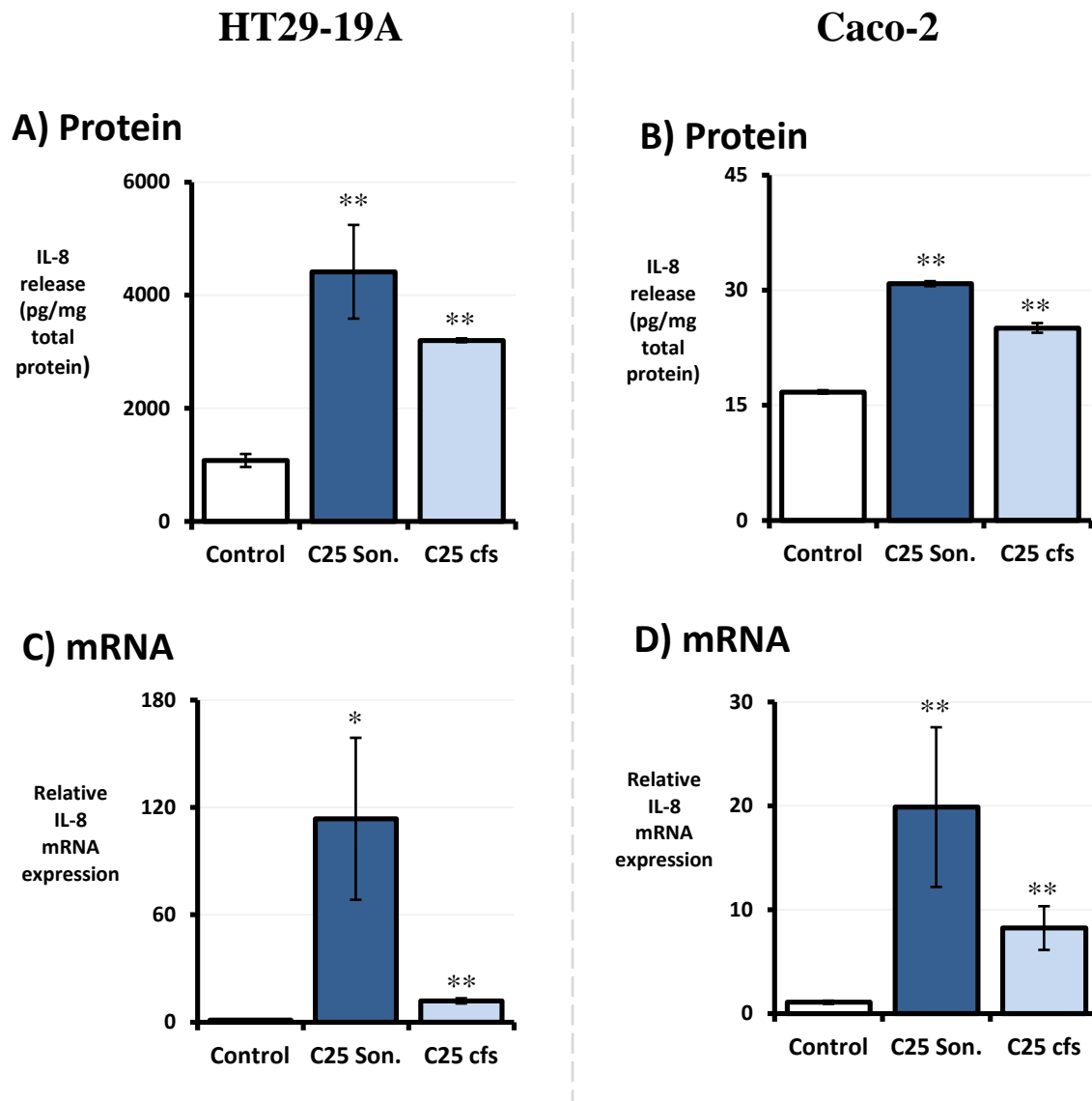


Figure 4.3 – *E. coli* C25 sonicates up-regulate IL-8 mRNA expression and secretion in IECs. IECs were challenged with a 1 in 10 dilution of sonicated *E. coli* C25 preparation (C25 Son.) or C25 cell-free supernatant (cfs). IL-8 release (**A** and **B**) and mRNA expression (**C** and **D**) were measured. Results are mean \pm SEM, n=3.

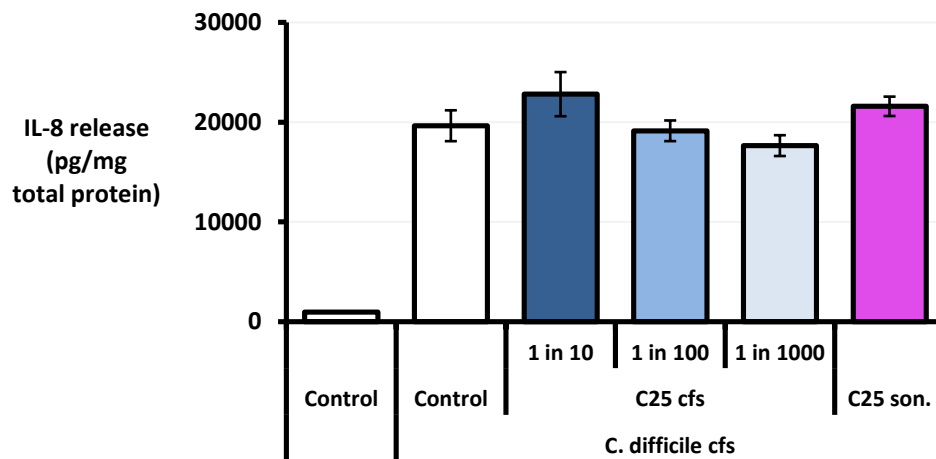
Gene	Fold difference in mRNA	
	HT29-19A	Caco-2
TLR-1	21.36 ± 12.46	11.05 ± 4.11**
TLR-2	188.20 ± 88.29*	3.21 ± 1.19*
TLR-4	43.74 ± 21.01*	28.74 ± 10.90**
TLR-9	ND	ND
CD14	0.99 ± 0.39	0.43 ± 0.15*

Table 4.2 – *E. coli* C25 sonicates differentially modify mRNA expression of TLRs in IECs. Both cell lines were challenged with a 1 in 10 dilution of *E. coli* C25 sonicates for 24 h and expression of TLR and CD14 mRNAs were investigated. Results were normalised to the housekeeping gene mRNAs, expressed as a fold difference compared to control cells and are mean ± SEM, n = 3. ND = not detected.

4.4 Extracellular products from *E. coli* C25 do not protect IECs against the cytotoxic effects of *C. difficile* cell-free supernatant

It was investigated whether or not the extracellular products from C25 predisposed the cell lines to any variance in response to challenge with *C. difficile* cell-free supernatant. Cells were pre-treated with 1 in 10 to 1 in 1000 dilutions of C25 cfs or 1 in 10 dilution of C25 sonicate for 24 h and were subsequently challenged with a 1 in 10 dilution of *C. difficile* cfs for a further 24 h. No significant differences in IL-8 release were observed in any of the pre-treated cells of either cell line (Figure 4.4).

A) HT29-19A



B) Caco-2

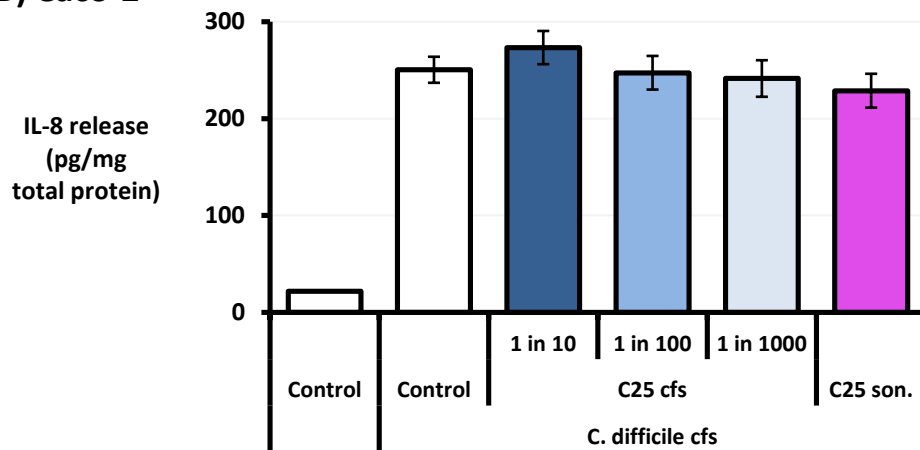


Figure 4.4 – Pre-treatment with *E. coli* C25 cell-free supernatant does not affect *C. difficile* cell-free supernatant-stimulated IL-8 in IECs. HT29-19A (A) and Caco-2 cells (B) were challenged with 1 in 10 to 1 in 1000 dilutions of *E. coli* C25 cell-free supernatant (C25 cfs) or a 1 in 10 dilution of sonicated *E. coli* C25 (C25 son.) for 24 h. Subsequently, supernatants were removed and cells were challenged with a 1 in 10 dilution of *C. difficile* cfs for 24 h. IL-8 release was analysed. Results are mean \pm SEM, n = 3.

4.5 Membrane vesicles (MVs) isolated from *E. coli* C25 elicit IL-8 expression in IECs

Both cell lines were challenged with a concentration range of MVs isolated from *E. coli* C25. HT29-19A cells produced significantly ($p \leq 0.05$) increased amounts of IL-8, in response to C25 MVs (Figure 4.5a), and were sensitive to the culture concentration (1x) of MVs. Cells challenged with a 1x MV concentration, elicited a ~1.5-fold increase in IL-8 release in comparison to control cells (2234 ± 291.6 compared to 1491 ± 236.1 pg/mg total protein). At a 5x MV concentration, HT29-19A cells appeared to reach saturation point, with no further significant increases in IL-8 release observed, however, maximal release of IL-8 was seen in response to the 10x MV concentration (6347 ± 523.0 pg/mg). Caco-2 cells also responded to C25 MVs, however, they were markedly less sensitive than HT29-19A cells. A 5x MV concentration was required to stimulate a significant ($p \leq 0.05$) increase of IL-8 in Caco-2 cells (55 ± 9.3 compared to 15 ± 7.9 pg/mg total protein). Saturation appeared not to be reached in the MV concentration range tested and maximal IL-8 release (125 ± 19.2 pg/mg total protein) was reached at a 25x MV concentration (Figure 4.6a). The proinflammatory effects of C25 MVs was also demonstrated at the transcriptional level as both cell lines demonstrated a significant ($p \leq 0.01$) increase in IL-8 mRNA in response to C25 MVs. In HT29-19A cells, a 10x MV concentration of gave a ~5.6-fold increase in IL-8 mRNA (Figure 4.5b), whereas in Caco-2 cells, a 25x concentration elicited a ~2.1-fold increase (Figure 4.6b).

In response to a 10x MV concentration, HT29-19A cells exhibited a small, but statistically significant ($p \leq 0.01$), increase in transcriptional expression of TLR-4, with a ~1.3-fold increase evident (Table 4.3). Additionally, there was a suggestion of up-regulation of TLR-2 and NOD1 mRNAs (~4.9- and ~1.5-fold increases, respectively; Table 4.3); however, due to variance of results, these were not statistically significant. Conversely, MV-challenged HT29-19A cells demonstrated a significant decrease in TLR-9 mRNA expression,

with no detectable levels present. There was a suggestion that TLR-9 mRNA was up-regulated in Caco-2 cells, in response to a 25x MV concentration, with a ~1.9-fold increase observed, however, this result was not statistically significant. Additionally, in Caco-2 cells, the mRNA levels of TLR-4 and CD14 were found to be significantly ($p \leq 0.05$) decreased, with ~0.2- and ~0.8-fold down-regulations in expression, respectively (Table 4.3).

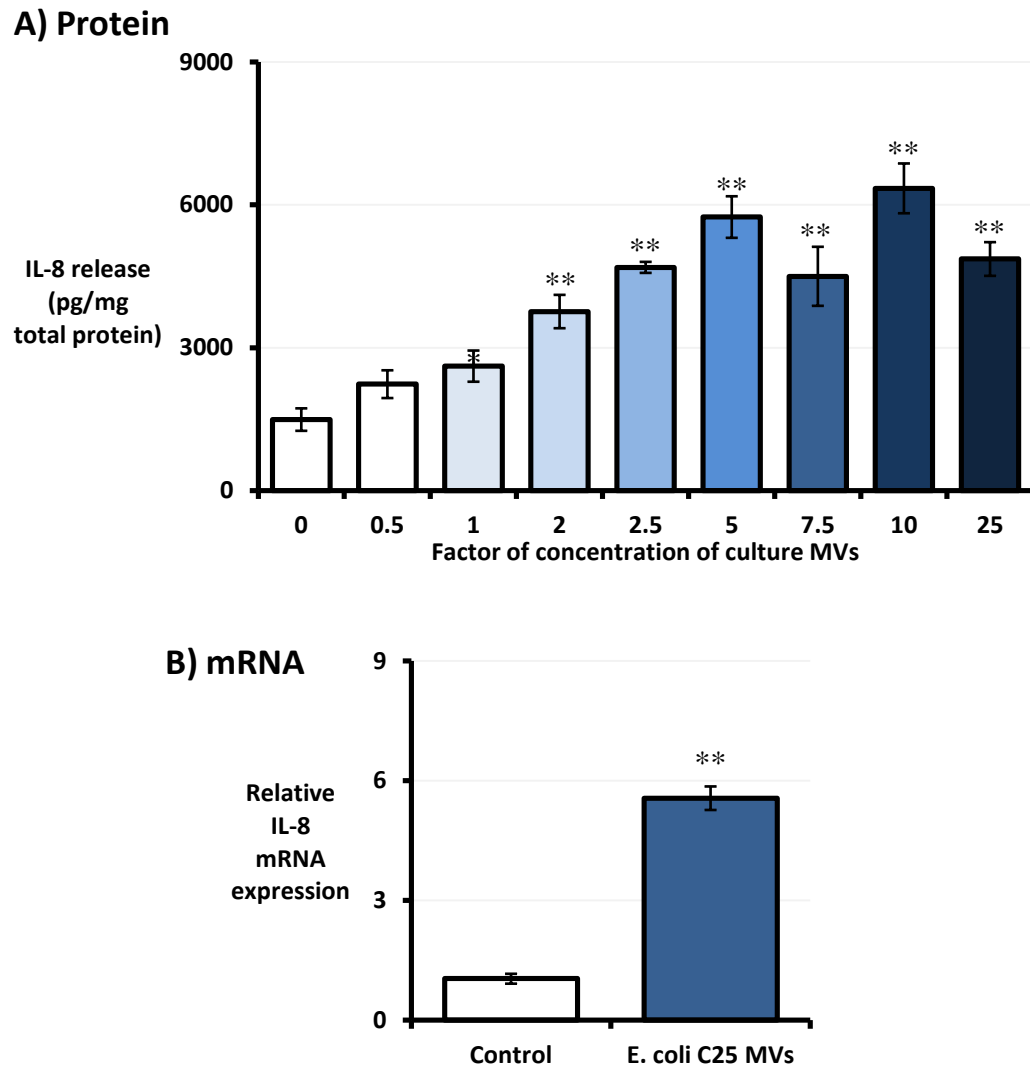


Figure 4.5 – *E. coli* C25 MVs stimulate IL-8 expression in HT29-19A cells. HT29-19A cells were challenged with serial dilutions (0.5-25x) of *E. coli* C25 MVs for 24 h and IL-8 release was measured (**A**). In cells, challenged with 10x MVs, IL-8 mRNA expression was investigated (**B**). Results are mean \pm SEM, n = 3-6.

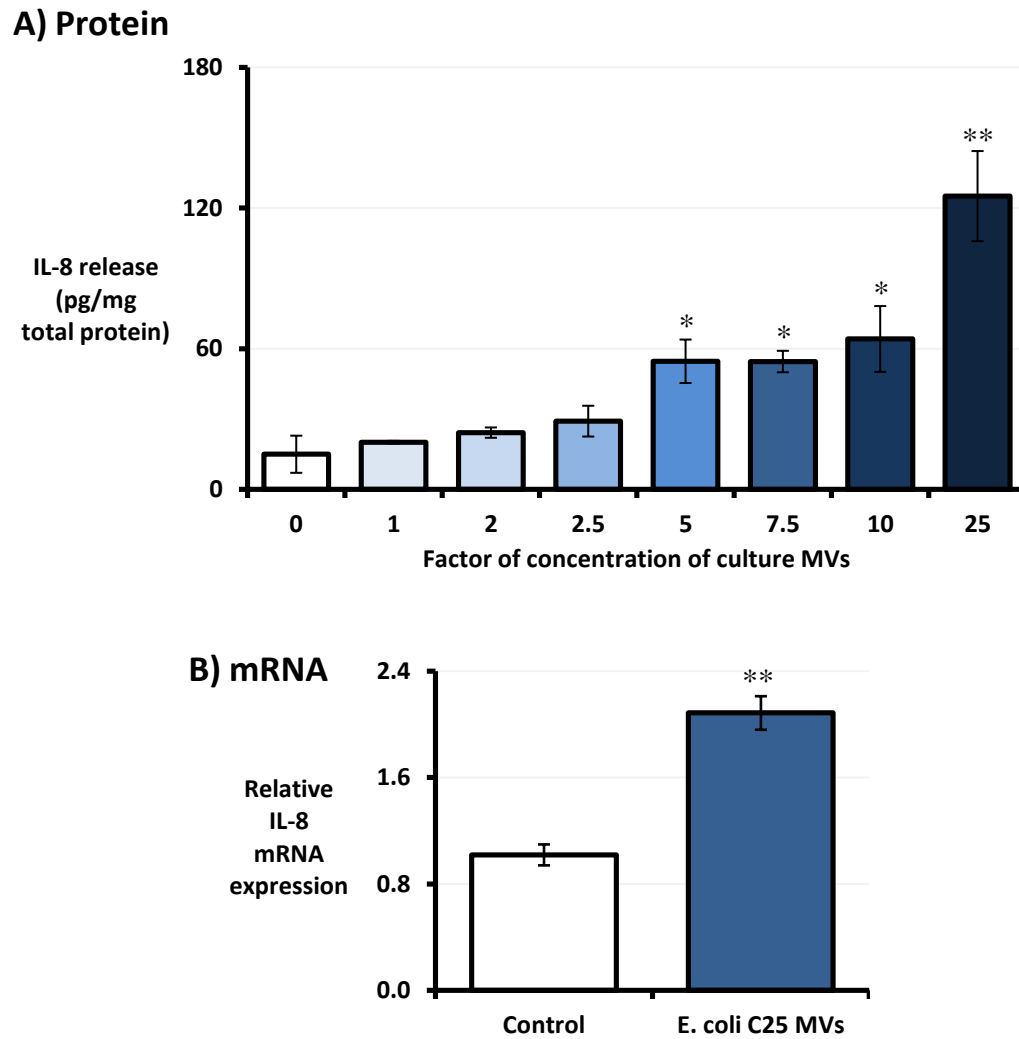


Figure 4.6 – *E. coli* C25 MVs stimulate IL-8 expression in Caco-2 cells. Caco-2 cells were challenged with serial dilutions (1-25x) of *E. coli* C25 MVs for 24 h and IL-8 release was measured (**A**). In cells, challenged with 10x MVs for 24 h, IL-8 mRNA expression was investigated (**B**). Results are mean \pm SEM, n = 3-6.

Gene	Fold difference in mRNA	
	HT29-19A	Caco-2
TLR-1	0.94 ± 0.06	0.77 ± 0.07
TLR-2	4.93 ± 1.97	0.84 ± 0.06
TLR-4	1.29 ± 0.06**	0.21 ± 0.08*
TLR-9	ND	ND
CD14	1.26 ± 0.13	0.78 ± 0.08*
NOD1	1.50 ± 0.16	1.03 ± 0.05

Table 4.3 – TLR expression modulation, in IECs, in response to MVs isolated from *E. coli* C25. Epithelial cells were challenged with a 10x culture concentration of *E. coli* C25 MVs for 24 h and PRR mRNA expression was measured. Results are mean ± SEM, n = 3. ND = not detected.

4.6 *E. coli* C25 LPS stimulates IL-8 release from HT29-19A cells

LPS was isolated from *E. coli* C25 and its antigenicity was tested against the two intestinal epithelial cell lines. HT29-19A cells were sensitive to C25 LPS at all concentrations tested and consequently displayed a significant ($p \leq 0.01$) increase in IL-8 release, when compared to control cells (Figure 4.7a). Additionally, a threshold level of value of 100 ng/ml was observed. In contrast, Caco-2 cells were unresponsive to C25 LPS, with no significant change in IL-8 release evident, in reaction to ≤ 1000 ng/ml LPS (data not shown). Neither cell line showed any detectable release of IL-10 in response to C25 1000 ng/ml LPS.

The proinflammatory activity of C25 LPS with HT29-19A cells was also observed at the transcriptional level, with a 5.1-fold increase ($p \leq 0.01$) in IL-8 mRNA, when compared to control level expression (Figure 4.7b). However, exposure to the commensal-derived LPS did not cause any significant differences in TLR-4 (Figure 4.7c), or its co-factor, CD14 (Figure 4.7d), at the transcriptional level.

The stimulatory effects of LPS on HT29-19A cells were shown to require the presence of serum (FBS), as, in its absence, no significant difference in IL-8 release was observed (Figure 4.8). Additionally, in the absence of serum, the stimulatory activity of 1 in 10 and 1 in 100 dilutions of *E. coli* C25 cell-free supernatant, on HT29-19A cells, was significantly lower than in its presence (Figure 4.9). This effect was lost at the 1 in 1000 dilution.

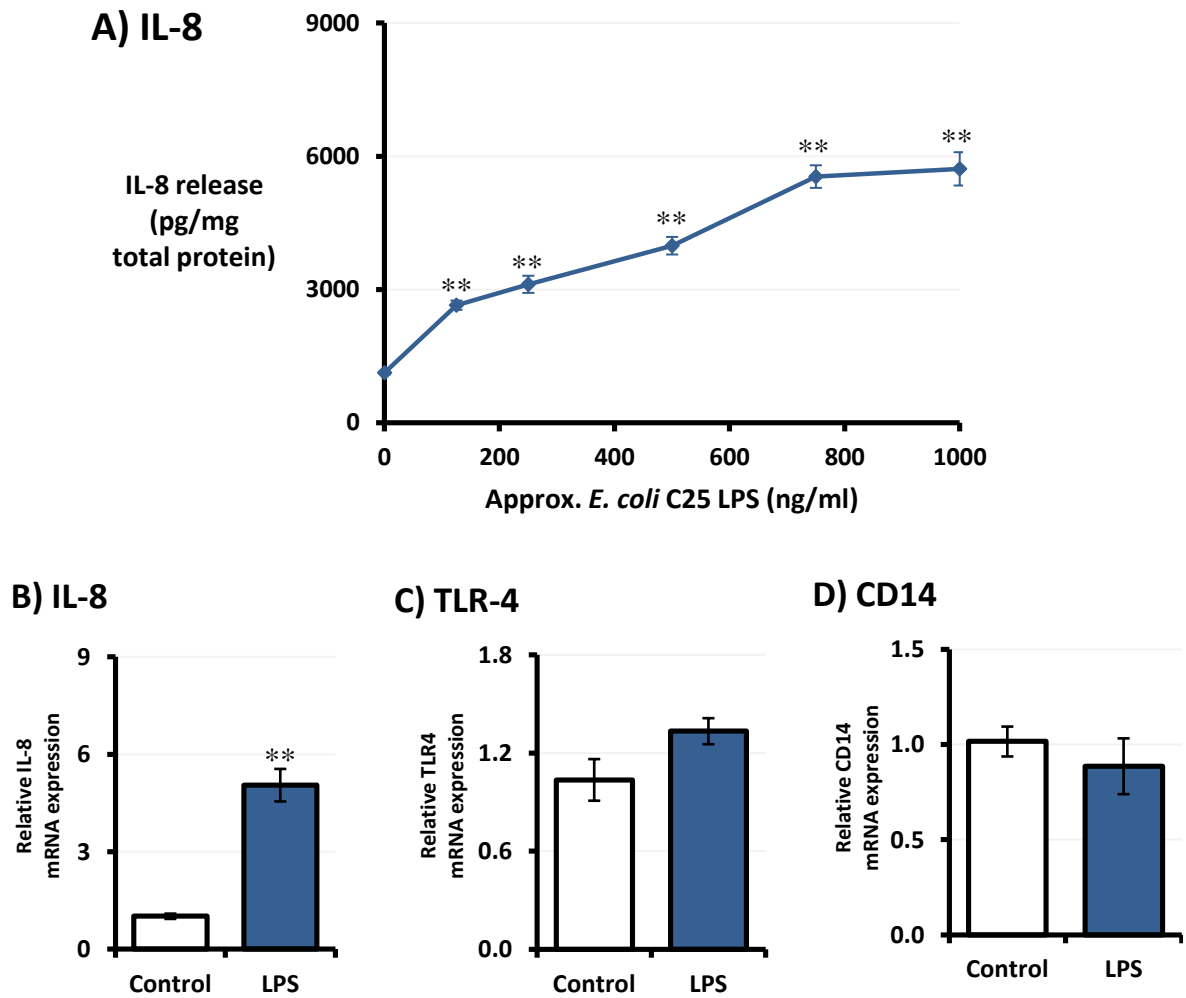


Figure 4.7 –C25-derived LPS stimulates increased IL-8 at both the transcriptional and translational level, in HT29-19A cells. LPS was isolated from *E. coli* C25 cultures at approximately 30 µg/ml. HT29-19A cells were challenged with 100-1000 ng/ml LPS for 24 h and IL-8 release (**A**) was determined. Additionally, in cells challenged with 1000 ng/ml LPS, mRNA expression of IL-8 (**B**), TLR-4 (**C**) and CD14 (**D**) was analysed. Results are mean ± SEM, n = 3.

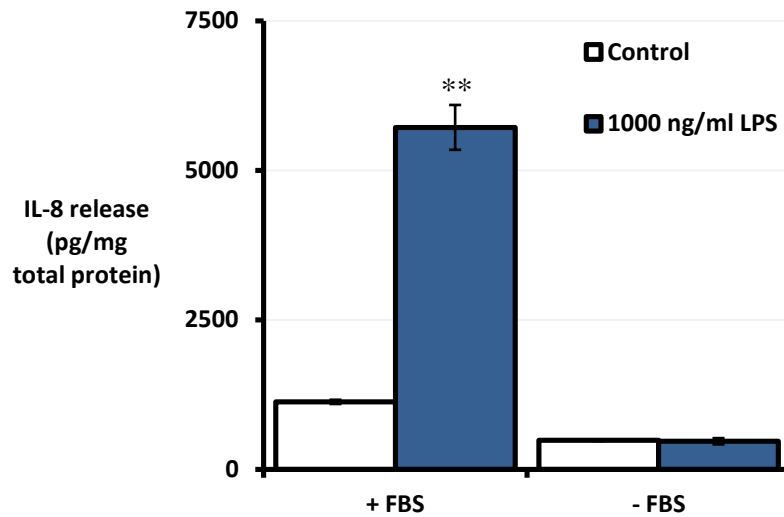


Figure 4.8 – LPS requires the presence of serum to exact its activity on HT29-19A cells. HT29-19A cell layers were cultured to confluence, challenged with 1000 ng/ml LPS in the presence (+) and absence (-) of serum (FBS), for 24 h and IL-8 levels were measured. Results are mean \pm SEM, n = 3-6.

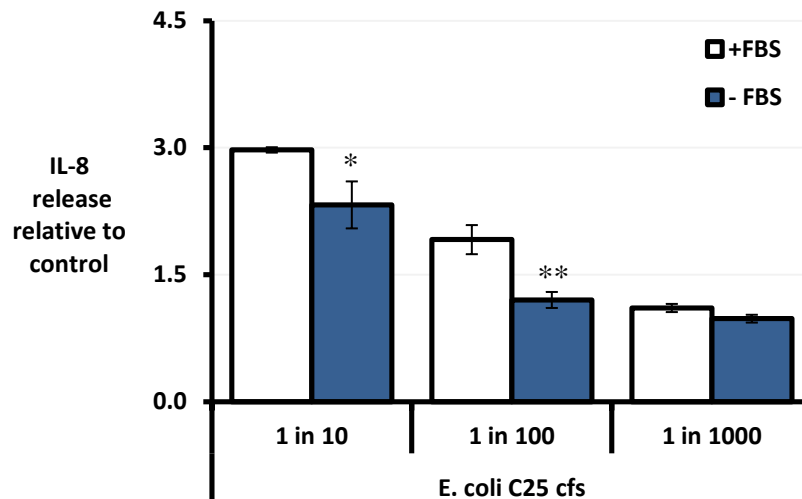
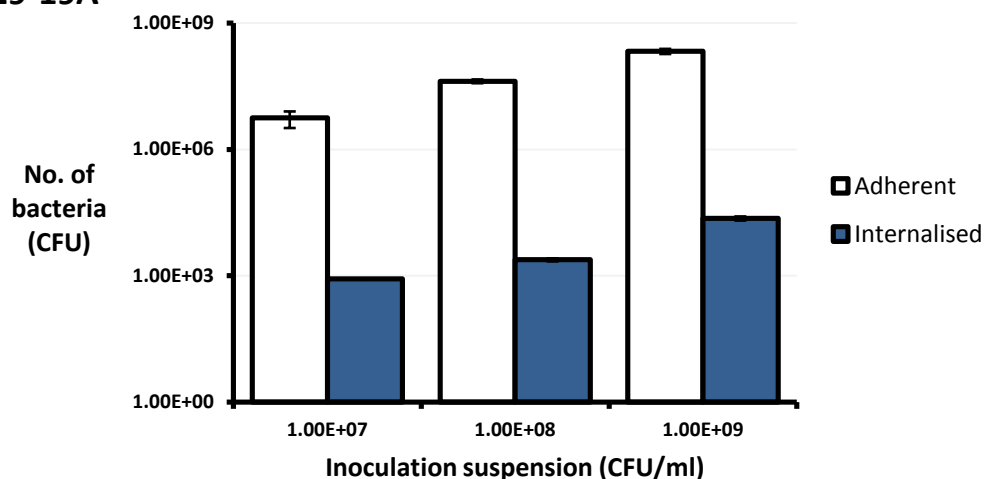


Figure 4.9 – LPS is a significant contributor to the immunomodulatory activity of C25 extracellular products. HT29-19A cell layers were challenged with 1 in 10 to 1 in 1000 dilutions of *E. coli* C25 cfs, in the presence (+) or absence (-) of FBS, for 24 h and IL-8 release was measured. Results are mean \pm SEM, n = 6.

4.7 *E. coli* C25 does not acutely modulate its own adherence or internalisation in IECs

In both HT29-19A and Caco-2 cell lines, adherence and internalisation increased proportionally to the number of bacteria in the original inoculum (Figure 4.10). However, when the numbers of bacteria adhered or internalised were expressed as a percentage of the original inoculum, it was observed that the increasing numbers of bacteria had no significant modulatory effects (Table 4.4).

A) HT29-19A



B) Caco-2

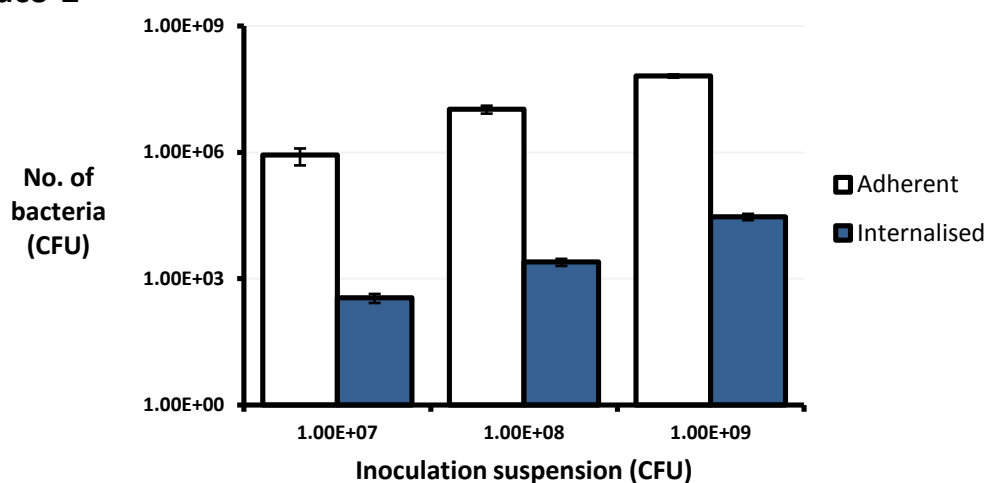


Figure 4.10 – Adherence and internalisation of *E. coli* C25 increase relative to the inoculum bacterial density. HT29-19A (A) and Caco-2 (B) cell layers were cultured to confluence and challenged with inocula of varying bacterial densities for 4 h. Adherent and internalised bacteria were plated out and counted. Conversely, non-internalised bacteria were killed off by washing with 50 µg/ml gentamicin and internalised bacteria were plated out and counted. Results are mean ± SEM, n = 3-6.

Original suspension (CFU/ml)	Adherence (% of orig. sus.)		Internalisation (% of orig. sus.)	
	HT29-19A	Caco-2	HT29-19A	Caco-2
1.00E+07	33.73 ± 11.57	8.63 ± 3.73	0.0028 ± 0.0008	0.0029 ± 0.0008
1.00E+08	37.53 ± 1.65	10.57 ± 2.25	0.0024 ± 0.0002	0.0025 ± 0.0005
1.00E+09	31.81 ± 4.38	6.50 ± 0.57	0.0023 ± 0.0003	0.0030 ± 0.0005

Table 4.4 – *E. coli* C25 does not acutely promote its own adherence or internalisation.

Cell layers were cultured to confluence and co-cultured with $\sim 1 \times 10^9$ cfu/ml cultures of *E. coli* C25 for 4 h. Adherent and internalised bacteria were plated out and counted. Conversely, non-internalised bacteria were killed off by washing with 50 µg/ml gentamicin and internalised bacteria were plated out and counted. Results were expressed as a % of the original bacterial inoculum and were normalised to the control. Results are mean ± SEM, n = 3-6.

4.8 Extracellular products from *E. coli* C25 have differential effects on bacterial internalisation

Having shown the immunostimulatory effects of extracellular products from *E. coli* C25 on epithelial cells (see Sections 4.2, 4.3, 4.4 and 4.5), their effects on the internalisation of the bacterium were considered. Epithelial cells were pre-treated with the various extracellular products for 24 h and subsequently subjected to the translocation assay (see Section 2.7).

Following pre-treatment with a 1 in 10 dilution of *E. coli* C25 cfs, both HT29-19A and Caco-2 cells, demonstrated significant ($p \leq 0.01$) increases in the numbers of bacteria undergoing internalisation, with 1.6- and 1.9-fold increases observed, respectively (Figure 4.11). In Caco-2 cells, pre-treatment with a 1 in 10 dilution of C25 sonicate was able to induce a significant (4.1-fold; $p \leq 0.01$) increase in internalisation of C25 (Figure 4.11b). Also, pre-treatment of HT29-19A cells with C25 sonicate appeared to modulate internalisation, as a 2.2-fold increase was evident; however, variance between replicates meant this result was not statistically significant (Figure 4.11a). Conversely, pre-treatment with a 10x MV concentration significantly ($p \leq 0.05$) inhibited bacterial internalisation, giving a 70 % decrease in the HT29-19A cell line (Figure 4.11a) and a 30 % decrease in Caco-2 cells (Figure 4.11b). Pre-treatment with *E. coli* C25 LPS did not exhibit any significant effects on bacterial internalisation in either cell line (Figure 4.11).

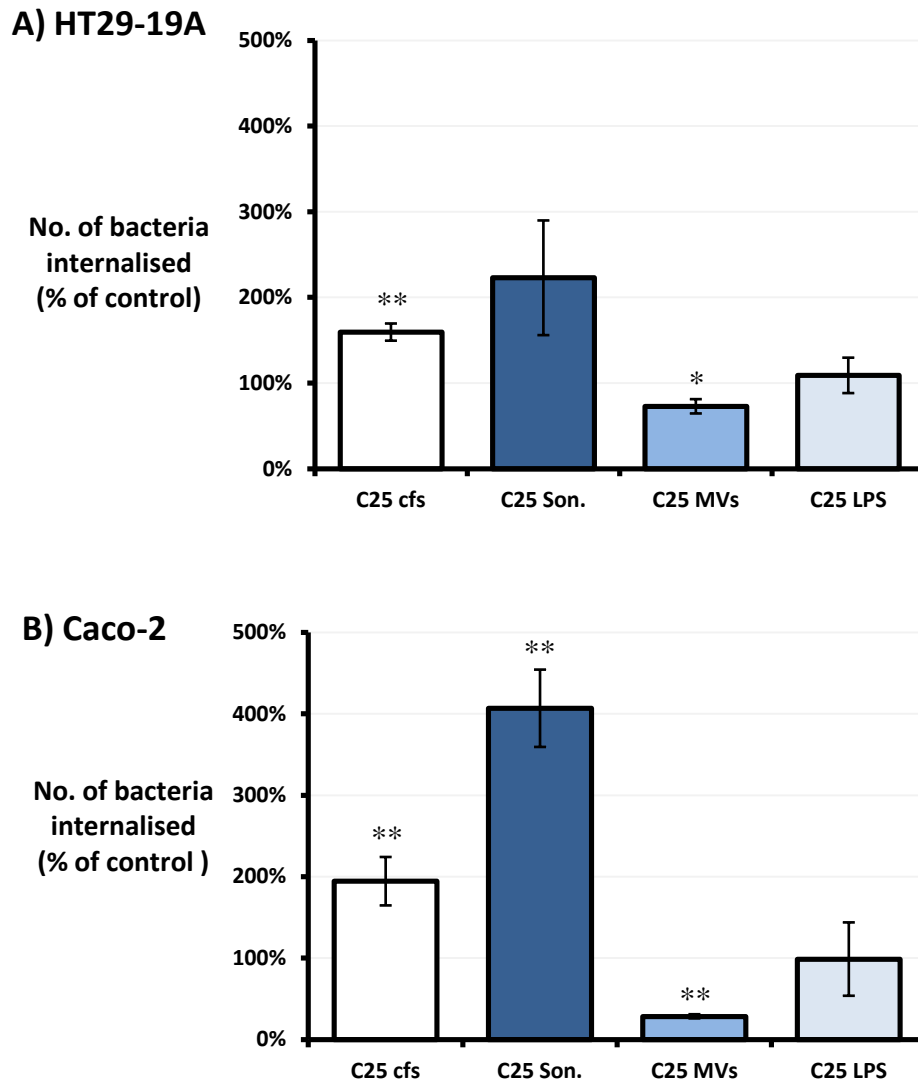


Figure 4.11 – *E. coli* C25 extracellular products differentially modify bacterial internalisation in IECs. HT29-19A (A) and Caco-2 (B) cells were challenged with the various extracellular products isolated from *E. coli* C25 for 24 h. Subsequently, the supernatants were removed and cell layers were co-cultured with $\sim 1 \times 10^9$ CFU of *E. coli* C25 for 4 h. Non-internalised bacteria were killed and epithelial cells were lysed, releasing internalised bacteria. Lysates were serially diluted, plated out and incubated for 24h. Colonies were counted and expressed as a % of the original inoculum. Results are mean \pm SEM, n = 4-6.

4.9 Summary

Stimulation of both HT29-19A and Caco-2 cells with cell-free supernatant (cfs) from the Gram-negative, commensal bacterium, *E. coli* C25, caused a significant increase in the expression and release of the proinflammatory chemokine IL-8. C25 cfs was also shown to up-regulate the mRNA expression of several TLRs in both cell lines. Similar patterns were observed in response to C25 sonicates, as both cell lines again exhibited significant increases in expression and release of IL-8 and modulation of TLR mRNA expression. Interestingly, C25 cfs was also able to up-regulate mRNA expression of the potent anti-inflammatory mediator, IL-10, in both cell lines; however, this effect was not paralleled by actual release of the cytokine, as no detectable levels were apparent.

MVs isolated from cultures of *E. coli* C25 were shown to elicit IL-8 secretion from both cell lines, in a concentration-dependent manner; however, HT29-19A cells demonstrated a 5-fold higher sensitivity than Caco-2 cells. Stimulation of the proinflammatory response was mirrored at the transcriptional level, with significant increases in IL-8 mRNA exhibited, in both cell lines, in response to challenge with MVs. However, qRT-PCR analysis of the mRNA expression of PRRs (TLRs-1, -2, -4, -5 and -9 and NOD1) following challenge with MVs showed no significant changes.

Caco-2 cells were unresponsive to *E. coli* C25 LPS at the concentration range tested, whilst HT29-19A cells were shown to elicit significant levels of IL-8, with increases in both transcriptional and translational expression observed. It was also found that the presence of serum was required for the antigenicity of LPS with HT29-19A cells, and its absence in the challenge of HT29-19A cells with C25 cfs caused a significant reduction in IL-8 secretion.

Having established the inflammatory activity of *E. coli* C25 extracellular products, their potential for modifying the internalisation of C25 was tested. It was found that C25 did not acutely modulate its own internalisation in control conditions; however, pre-treatment of

both cell lines, with C25 cfs, caused a significant increase in the number of bacteria internalised. Additionally, Caco-2 cells pre-treated with C25 sonicate demonstrated a ~4-fold increase in bacterial internalisation. Conversely, pre-treatment of both HT29-19A and Caco-2 cells with C25 MVs caused a significant decrease in internalisation.

In conclusion, these results indicate that the extracellular products of *E. coli* C25 have a proinflammatory effect on intestinal epithelial cells, with MVs and LPS both contributing.

Chapter 5

Epithelial interactions with uncharacterised strains of EPS-producing lactobacilli

5.1 Introduction

Lactobacilli are a large heterogeneous group of Gram-positive, non-sporulating, facultative anaerobes that belong to the Firmicutes phylum (Lebeer *et al.*, 2008, Wells, 2011). The lactobacillus genus is so-named as its members constitute cells of the bacilliform morphology that produce lactic acid as the major end-product in their metabolism of lactose and other fermentable carbohydrates (Slattery *et al.*, 2010). In addition to fermentable carbohydrates, lactobacilli require a complex mixture of amino acids, peptides, fatty acids, esters, salts, nucleic acids derivatives and vitamins to sustain their growth (Lebeer *et al.*, 2008). However, despite their fastidious nutritional needs, lactobacilli are found in a wide range of environmental niches, from plants, water, soil and sewage, to the commensal microflora of the human oral, intestinal and vaginal cavities (Stiles and Holzapfel, 1997). In the adult human intestine, lactobacilli are present in relatively low numbers, representing just 0.01-0.6 % of the total faecal bacteria (Lebeer *et al.*, 2008). However, their population can be artificially boosted, as lactobacilli are found in a wide range of fermented food products, such as milk drinks, cheeses and fruit juices, either as members of the natural bacterial content, or as commercially administered probiotic cultures (Turpin *et al.*, 2010). Lactobacilli are also well characterised in their production of EPSs (Badel *et al.*, 2010), which are increasingly utilised in food products (Marshall and Rawson, 1999, De Vuyst and Degeest, 1999).

Lactobacillus acidophilus, an enteric species of the lactobacillus genus, was originally isolated in the early 20th century, from the faeces of a healthy infant, and was thus named due its aciduric nature (Stiles and Holzapfel, 1997). Subsequently, *L. acidophilus* cultures have been heavily utilised as a starter cultures in the production of fermented food products, such as milk drinks (Gilliland, 1989, Stiles and Holzapfel, 1997) and yoghurts (Anderson and Gilliland, 1999), and a number of strains, such as NCFM (Sanders and Klaenhammer, 2001), M92 (Kos *et al.*, 2003) and LA1 (Bernet *et al.*, 1994, Bernet-Camard *et al.*, 1997) have

exhibited strong probiotic qualities. Subsequently, a closely related, yet non-enteric lactobacilli species, *Lactobacillus helveticus*, has been isolated from Emmental cheese (Slattery *et al.*, 2010), and is itself now widely used as a starter culture in the production of Emmental and a number of other cheeses (Slattery *et al.*, 2010).

L. acidophilus 5e2 (5e2) and *L. helveticus* sp. Rosyjski (Rosy) are two novel EPS-producing lactobacilli identified by a European Union International Scientific Cooperation Project (IC15-CT98-0905; <http://imol.vub.ac.be/IMDO/projects/EPSLAB.html>), that have only previously been regarded in the context of the chemical composition of their EPSs (Laws *et al.*, 2008, Leivers, 2011). The aim of this chapter was the investigation of the immunomodulatory activity of extracellular products, particularly EPSs which were isolated and ultrapurified from cultures of 5e2 and Rosy.

5.2 Crude lactobacilli cell-free supernatants stimulate a low level proinflammatory response in IECs

To test the immunomodulatory potential of the extracellular products from 5e2 and Rosy, the HT29-19A and Caco-2 intestinal epithelial cells lines were initially challenged with crude cell-free supernatants (cfs) and IL-8 and IL-10 release were investigated.

In HT29-19A cells, a 1 in 10 dilution of 5e2 cfs was able to elicit a statistically significant increase in IL-8 release (1.4-fold; $p \leq 0.01$), when compared to the MRS broth (vehicle) control (2033 ± 16.8 compared to 1442 ± 82.9 pg/mg total protein); however, the stimulatory effects were lost as the cfs reached dilutions of 1 in 100 and 1 in 1000 (Figure 5.1a). On the other hand, Rosy crude supernatant demonstrated no statistically significant effect on IL-8 production in HT29-19A cells (Figure 5.1b). Additionally, neither strain of *Lactobacillus* demonstrated any detectable stimulation of IL-10 in the HT2-19A cell line (data not shown).

In contrast to the minimal stimulatory effects seen in the HT29-19A cell line, Caco-2 cells appeared more responsive to the lactobacilli crude supernatants. A 1 in 10 dilution of 5e2 cell-free supernatant was able to elicit a 3.5-fold increased secretion of IL-8 when compared to levels in the MRS broth (vehicle) control in Caco-2 cells (222 ± 8.9 compared to 63 ± 1.2 pg/mg total protein), however; as in the HT29-19A cell line, the stimulatory effect was lost as the supernatant reached dilutions of 1 in 100 and 1 in 1000 (Figure 5.2a). Additionally, Rosy cell-free supernatant was able to potentiate the release of IL-8 ~3-fold (from the basal 41 ± 1.3 to 122 ± 3.5 pg/mg total protein), but, again, this activity was only evident at the 1 in 10 dilution level (Figure 5.2b). It is also worth noting that the MRS vehicle control media itself had a mild proinflammatory activity in the Caco-2 cell line at a 1 in 10 dilution, but this was lost in the 1 in 100 and 1 in 1000 dilutions of supernatant, as the level of IL-8 returned to that of the unchallenged control (Figure 5.2). Additionally, neither strain of

Lactobacillus was able to stimulate detectable levels of IL-10 release in Caco-2 cells (data not shown).

To investigate if the cytokine release was paralleled at the transcriptional level, the mRNA expression of IL-8 and IL-10 was investigated. Consistent with the IL-8 protein level expression, a 1 in 10 dilution of the crude supernatant from 5e2 stimulated a 2.5-fold increase in IL-8 mRNA in the HT29-19A cell line, whereas Rosy had no significant effect (Figure 5.3a). In the Caco-2 cell line, the transcriptional expression also mirrored the protein level expression with 2.3- and 2.5-fold increases in IL-8 mRNA in response to 1 in 10 dilutions of 5e2 and Rosy cfs, respectively (Figure 5.3b). Additionally, neither strain of *Lactobacillus* was able to stimulate the expression of IL-10 mRNA in HT29-19A cells with their cell-free supernatant, as expression remained undetectable in the 45 cycles of qRT-PCR (data not shown). Conversely, in the Caco-2 cell line, 5e2 cfs caused a significant decrease (0.2-fold: $p \leq 0.05$) in IL-10 mRNA expression, whereas its expression also remained unchanged in response to Rosy cfs (Figure 5.3c).

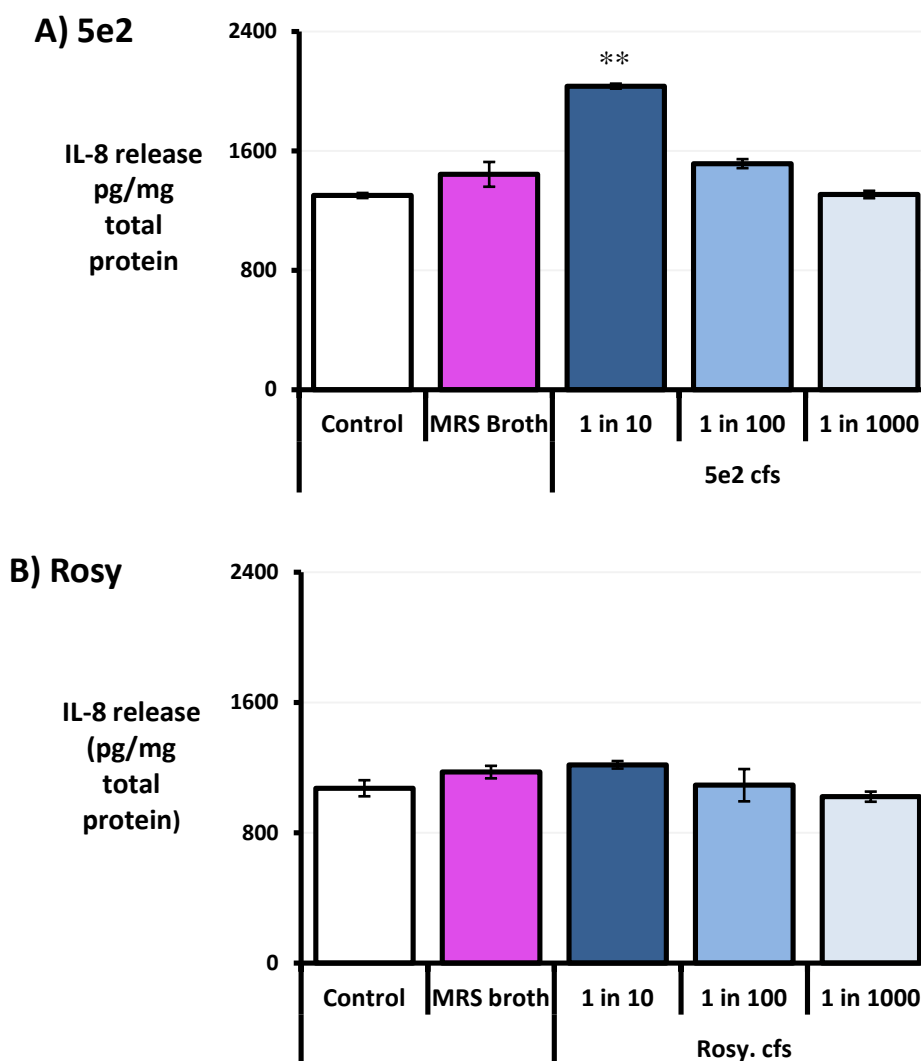


Figure 5.1 – *L. acidophilus* 5e2 cell-free supernatant stimulates IL-8 release in HT29-19A cells, but cell-free supernatant from *L. helveticus* sp. Rosyjski does not. HT29-19A cells were challenged with 1 in 10-1 in 1000 dilutions of cell-free supernatant, from cultures of *L. acidophilus* 5e2 (5e2; **A**) and *L. helveticus* sp. Rosyjski (Rosy; **B**), for 24 h. Cells were also challenged with a 1 in 10 dilution of MRS broth (vehicle control) and IL-8 release was measured. Results were normalised to total protein. Results are mean \pm SEM, n = 3.

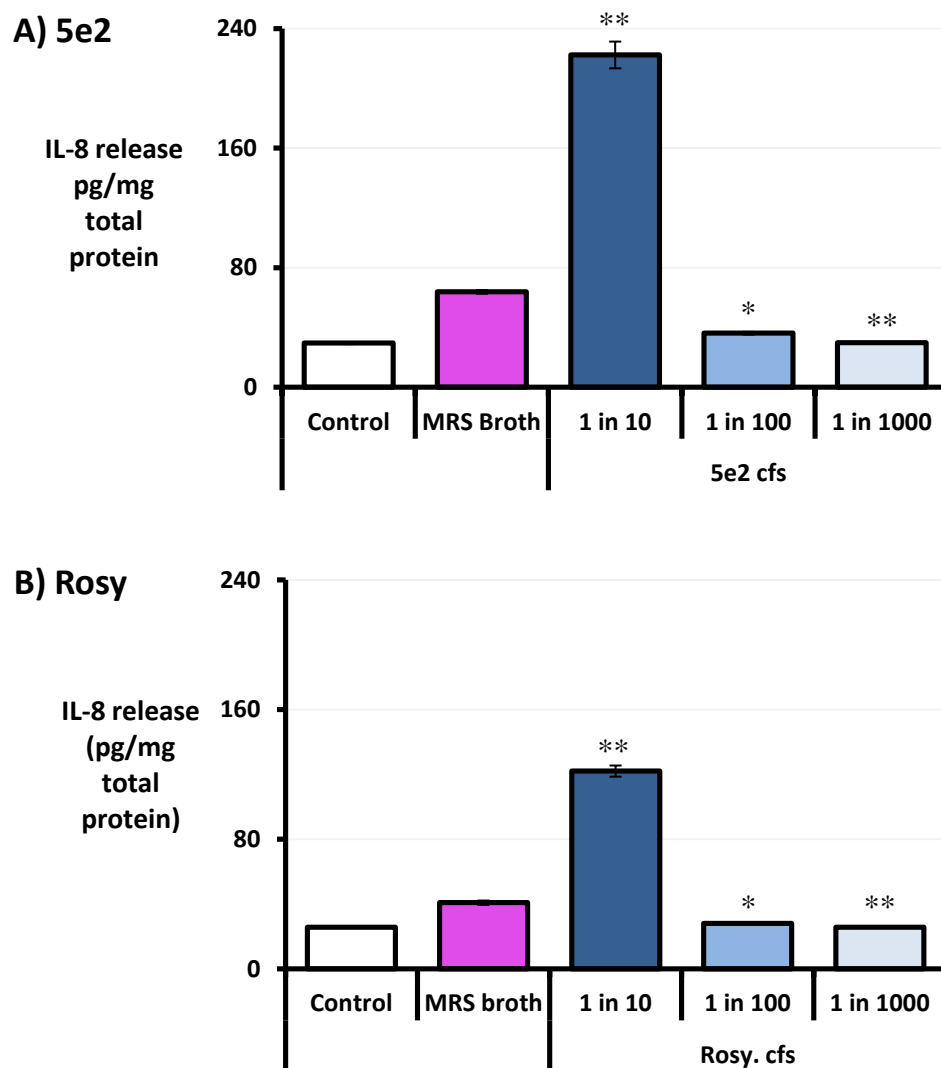


Figure 5.2 – Lactobacilli extracellular products elicit IL-8 from Caco-2 cells. Caco-2 cells were challenged with 1 in 10 to 1 in 1000 dilutions of cell-free supernatant from 5e2 (A) and Rosy (B) and a 1 in 10 dilution of the MRS broth vehicle control for 24 h. Supernatants were collected and analysed for IL-8. Result are mean \pm SEM, n = 3.

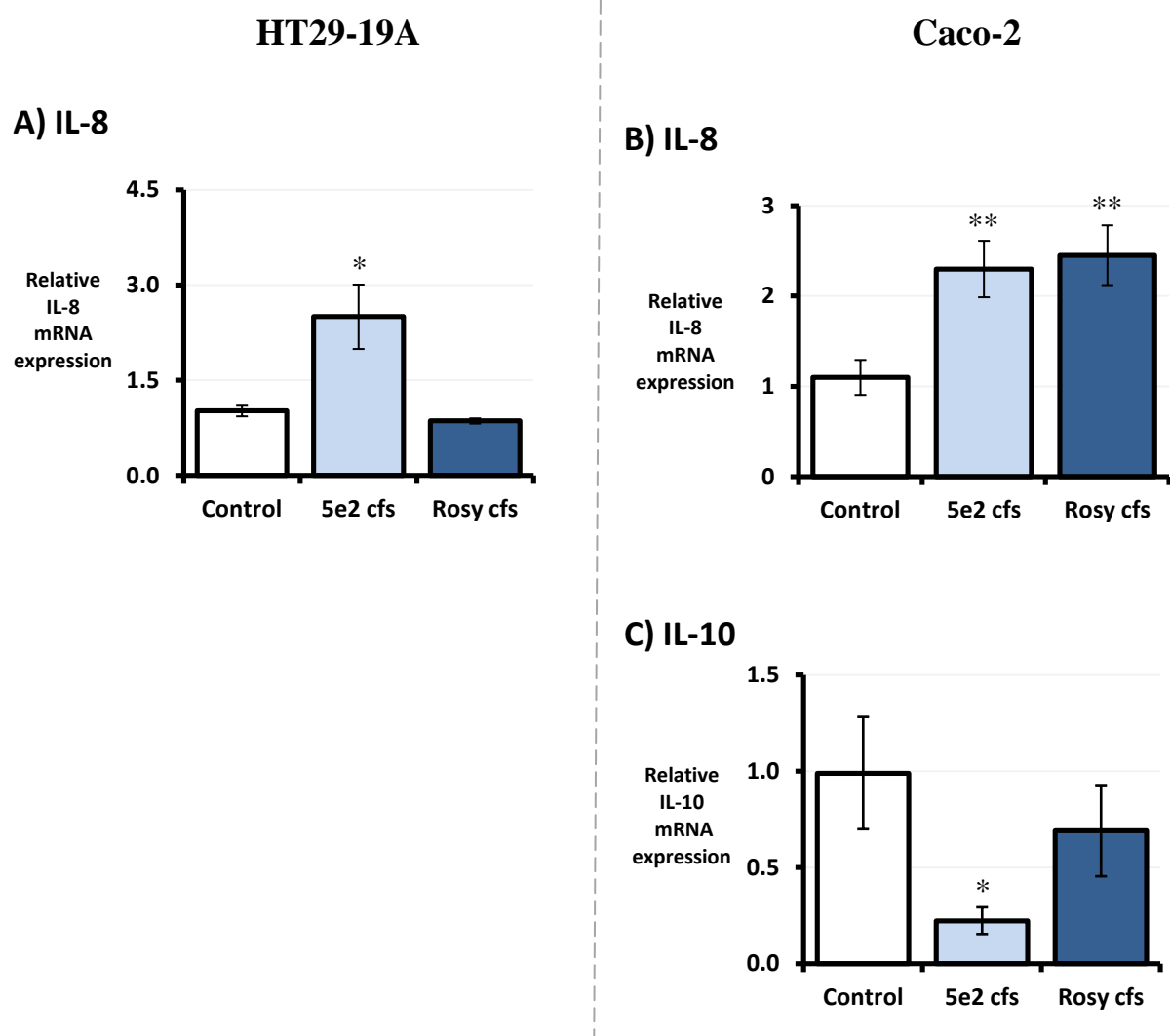


Figure 5.3 – IL-8 up-regulation in response to lactobacilli cfs is also shown at the transcriptional level. IECs were challenged with a 1 in 10 dilution of 5e2 or Rosy cfs, and a 1 in 10 dilution of the vehicle control (MRS broth) (Control) for 24 h. mRNA expression levels of IL-8 (**A** & **B**) and IL-10 (**C**) were measured. Results were normalised to housekeeping genes and are expressed as mean \pm SEM, $n = 3$.

5.3 Lactobacilli sonicates have an immunomodulatory effect on Caco-2 cells, but not with HT29-19A cells

The HT29-19A cell line did not exhibit any significant changes in IL-8 release in response to 1 in 10 dilutions of the lactobacilli sonicates (Figure 5.4). In contrast to this, both 5e2 (Figure 5.5a) and Rosy (Figure 5.5b) sonicates elicited significant ($p \leq 0.01$) increases in IL-8 release from Caco-2 cells, in comparison to vehicle control cells (4.2- and 3.4-fold increases, respectively). The proinflammatory effects on Caco-2 cells were also mirrored at the transcriptional level, with both sonicates triggering a significant up-regulation of IL-8 mRNA expression (4.4- and 11.9-fold, respectively; $p \leq 0.01$) (Figures 5.5c & 5.5d). When compared to the effects of cfs's, sonicates from both strains of lactobacilli exhibited greater proinflammatory activity on Caco-2 cells, with higher levels of IL-8 secretion evident (Figure 5.5).

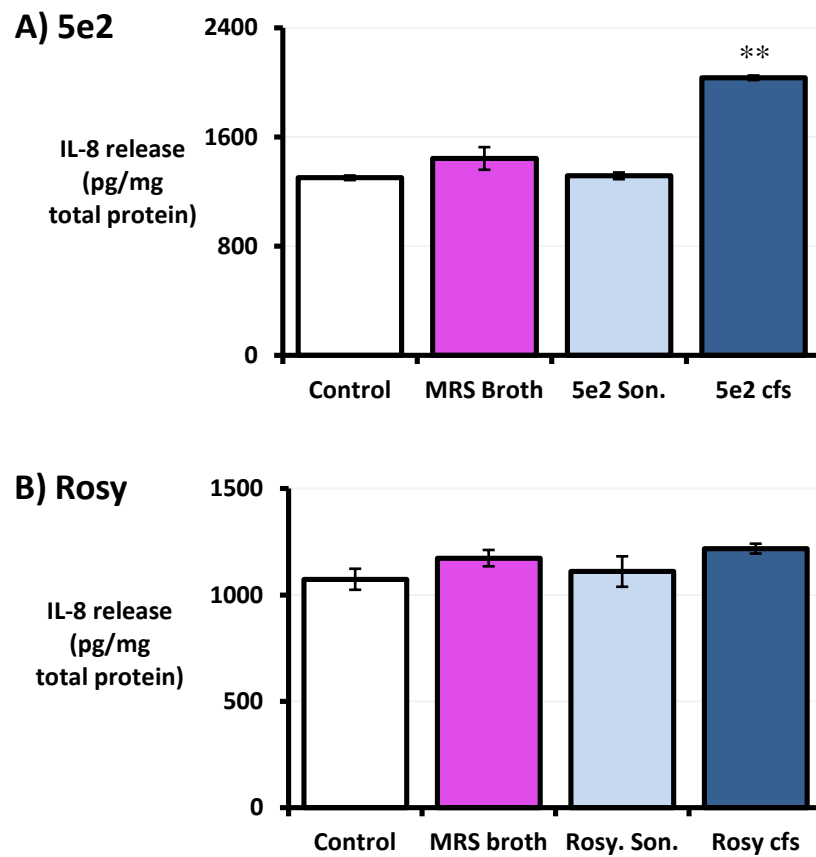


Figure 5.4 – HT29-19A cells are unresponsive to lactobacilli sonicates. HT29-19A cell layers were challenged with 1 in 10 dilutions of 5e2 (**A**) and Rosy (**B**) sonicates and MRS broth vehicle control for 24 h and IL-8 secretion was measured. Results are mean \pm SEM, n = 3.

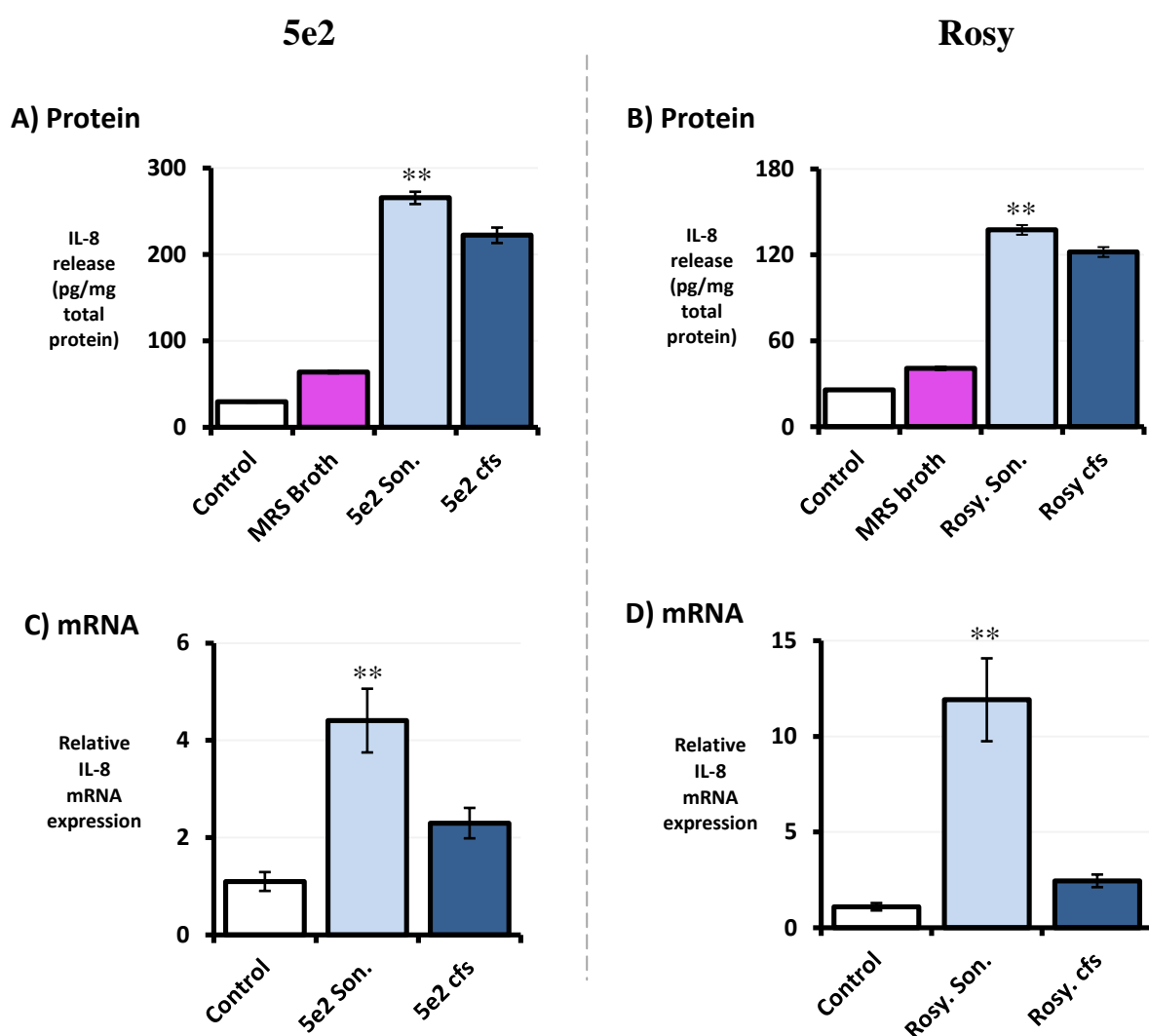


Figure 5.5 – Lactobacilli sonicates stimulate IL-8 expression in Caco-2 cells. Epithelial cells were challenged with a 1 in 10 dilution of 5e2 or Rosy sonicates and a 1 in 10 dilution of the MRS vehicle control for 24 h. IL-8 release (A, C) and mRNA expression (B, D) were analysed. Results are mean \pm SEM, n = 3.

5.4 Lactobacilli extracellular products differentially alter TLR expression in HT29-19A cells

Incubation with 5e2 cfs induced a significant ($p \leq 0.01$) potentiation of TLR-1 and TLR-2 mRNA expression in HT29-19A cells. In contrast, the mRNA expression of TLR-9 was considerably attenuated to undetectable levels in 45 cycles of qRT-PCR. However, there were no demonstrable effects on TLR-4 and TLR-5 mRNA (Table 5.1).

Challenge of HT29-19A cells with Rosy cfs also resulted in a significant ($p \leq 0.01$) up-regulation of TLR-2 mRNA, however, TLR-1, TLR-4 and TLR-5 exhibited no significant change in expression (Table 5.1). As was observed in response to 5e2 EPS, expression of TLR-9 mRNA was significantly ($p \leq 0.01$) attenuated, exhibiting down-regulation to such an extent that no detectable levels were present (Table 5.1).

mRNA expression of TLRs in the Caco-2 cell line remained largely unchanged by the presence of the crude supernatants from either strain of lactobacilli, with the exception of TLR-9 mRNA which showed significantly ($p \leq 0.01$) decreased mRNA expression levels in response to 5e2 cfs, (Table 5.1). Additionally, in response to a 1 in 10 dilution of 5e2 sonicate, Caco-2 cells exhibited a statistically significant ($p \leq 0.05$) augmentation of TLR-2 mRNA, when compared to expression in control cells (Table 5.1). TLR-1 mRNA also appeared to be up-regulated; however, variance between the replicates meant that the result was not statistically significant from the control. Conversely, TLR-9 mRNA was significantly down-regulated ($p \leq 0.01$), in comparison to constitutive expression. A 1 in 10 dilution of Rosy sonicate also had a regulatory effect on TLR mRNA expression in Caco-2 cells, with TLRs-1 and -2 both significantly ($p \leq 0.01$) up-regulated. TLR-9 expression showed a suggestion of down-regulation; however, the decrease was shown not to be statistically significant (Table 5.1).

Gene	Fold difference in mRNA					
	HT29-19A		Caco-2			
	5e2 cfs	Rosy cfs	5e2 cfs	Rosy cfs	5e2 son.	Rosy Son
TLR-1	5.09 ± 1.25**	0.97 ± 0.06	1.84 ± 0.41	0.81 ± 0.09	1.50 ± 0.20	5.86 ± 1.41**
TLR-2	148.92 ± 45.17**	29.98 ± 10.03*	1.02 ± 0.15	1.89 ± 0.78	1.54 ± 0.23*	2.01 ± 0.39**
TLR-4	1.22 ± 0.23	0.90 ± 0.04	1.54 ± 0.27	0.88 ± 0.11	-	-
TLR-5	0.85 ± 0.16	0.92 ± 0.05	1.29 ± 0.18	1.22 ± 0.13	-	-
TLR-9	ND	ND	0.27 ± 0.07**	0.83 ± 0.09	0.26 ± 0.03**	0.80 ± 0.28

Table 5.1 – Lactobacilli cell-free supernatants differentially modify TLR mRNA expression in IECs. IECs were challenged with 1 in 10 dilutions of cfs or sonicates (son.) from both 5e2 and Rosy, for 24 h and mRNA expression levels of TLRs were determined. Results were normalised to the β -actin and GAPDH housekeeping genes and are mean \pm SEM, n = 3. ND = not detected.

5.5 Pre-treatment of IECs with lactobacilli cfs does not protect against inflammatory response to *C. difficile* cfs

Pre-treatment of HT29-19A and Caco-2 cells with MRS broth (vehicle control), prior to challenge with a 1 in 10 dilution of *C. difficile* cfs, caused a statistically significant ($p \leq 0.05$) increase in IL-8 release, when compared to the release observed in cells with no pre-treatment (Control). However, pre-treatment with a 1 in 10 dilution of 5e2 cfs had no significant effect on IL-8 secretion, compared to the MRS vehicle control in either cell line (Figure 5.6). In contrast, Rosy cfs significantly ($p \leq 0.05$) attenuated IL-8 release in both cell lines, in response to *C. difficile* cfs, compared to the MRS vehicle control. Nevertheless, despite the reduction in IL-8, the release was still comparable to control cells that did not undergo pre-treatment (Figure 5.6).

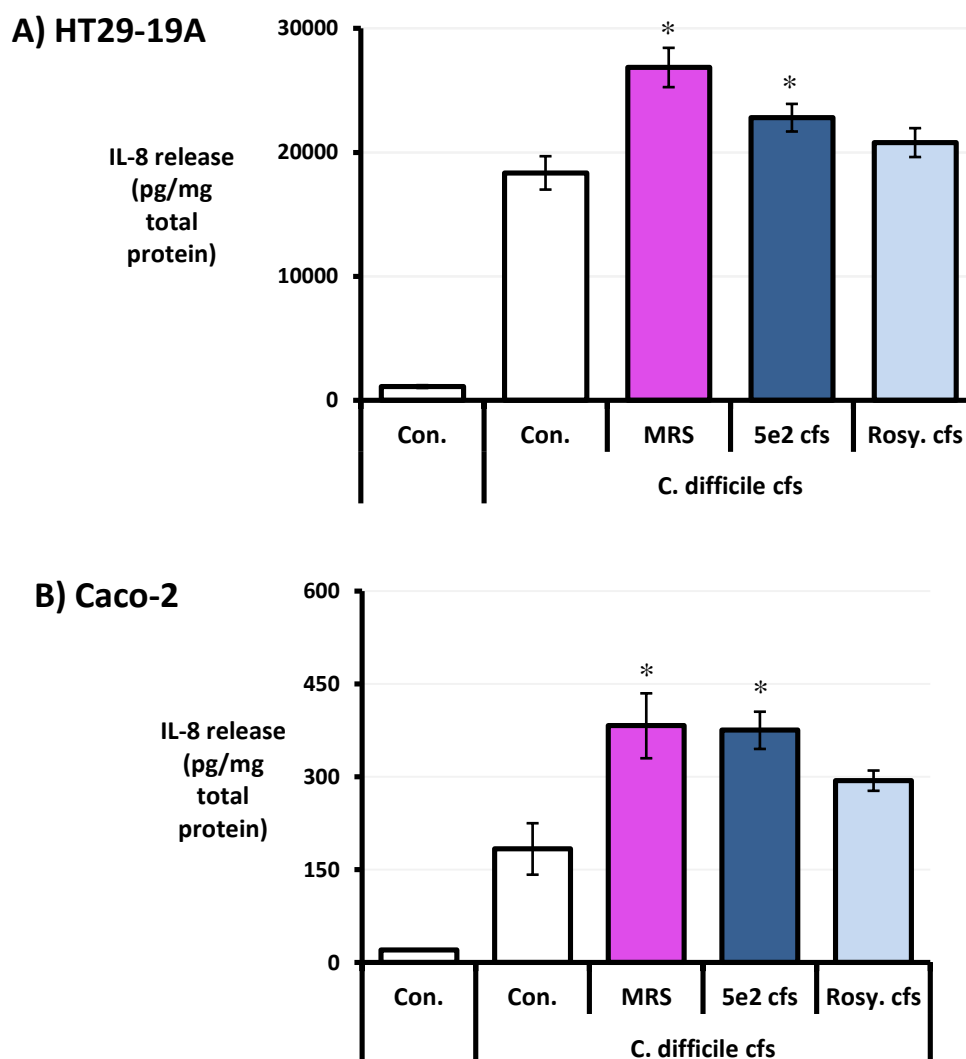


Figure 5.6 – Pre-treatment of IECs with lactobacilli cell-free supernatants do not protect against the inflammatory effects of *C. difficile* cell-free supernatant. HT29-19A (A) and Caco-2 (B) cells were pre-treated with 1 in 10 dilutions of 5e2 cfs, Rosy cfs, MRS broth (vehicle control) or cell culture media (control) for 24 h. Subsequently, the supernatant was removed and cells were challenged with a 1 in 10 dilution of *C. difficile* cfs for a further 24 h and IL-8 release was determined. Results are mean \pm SEM, n = 3.

5.6 *Lactobacillus* EPSs elicit IL-8 secretion from HT29 cells, but have no effect on IL-10

The immunomodulatory effects of EPSs isolated and purified from cultures of 5e2 and Rosy, on intestinal epithelial cells, were tested by challenging cells with 20 µg/ml EPSs for 24 h. In HT29-19A cells, both EPSs stimulated significant ($p \leq 0.01$) secretion of IL-8 (Figure 5.7a). Incubation with 5e2 EPS elicited a 1.6-fold rise in IL-8 secretion (2305.8 ± 250.1 pg/mg total protein) and Rosy EPS stimulated a 1.4-fold increase (2005.1 ± 138.4 pg/mg total protein), when compared to constitutive IL-8 release (1438.7 ± 133.4 pg/mg total protein). No deviation from the basal level of IL-8 was seen in Caco-2 cells (Figure 5.7b). No IL-10 release was detected from control or EPS-challenged cells, in both cell lines (data not shown).

HT29-19A cells showed a ~4-fold increase of IL-8 mRNA in response to both EPSs investigated (Figure 5.7c). Additionally, despite the effect not being reflected at the protein level, Caco-2 cells also exhibited a doubling of mRNA in response to 5e2 EPS (Figure 5.7d).

No detectable levels of IL-10 mRNA were observed in HT29-19A cells, in control or EPS-challenge experiments (data not shown). Additionally, no significant change in IL-10 mRNA was seen in Caco-2 cells in response to EPSs (Figure 5.7e).

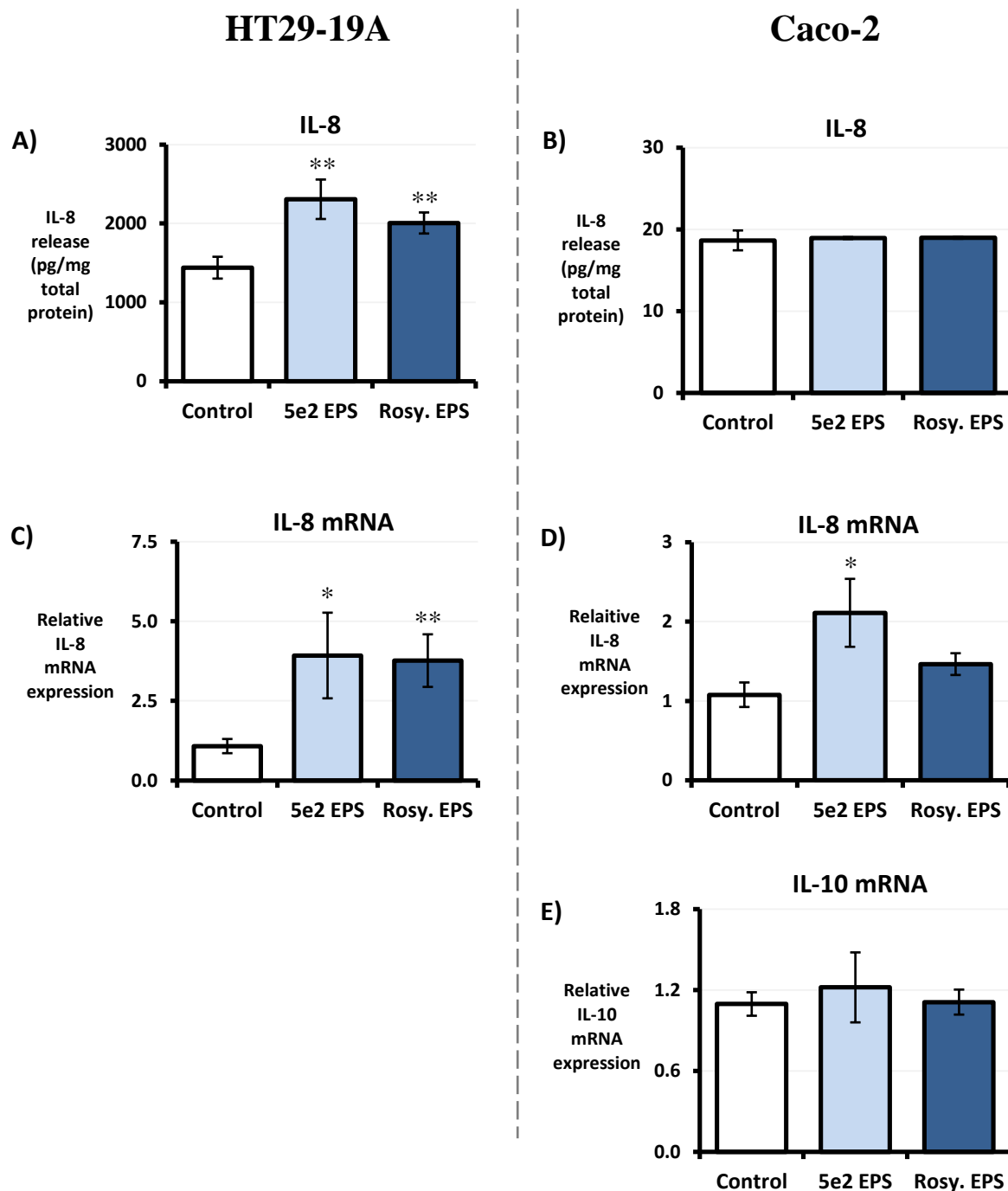


Figure 5.7 – Modification of inflammatory cytokine release and expression, in IECs, by EPSs from *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski. IECs were challenged with 20 µg/ml EPSs for 24 h and IL-8 (A, B) and IL-10 (data not shown) release were measured. mRNA expression of IL-8 (C, D) and IL-10 (E) were also explored. Results are mean ± SEM, n = 3-6.

5.7 *Lactobacillus* EPSs modify the mRNA expression of TLRs in IECs

Modifications in mRNA expression of TLRs-1, TLR-2, TLR-4, TLR-5 and TLR-9, in response to EPSs, were explored. TLR-1 (Figure 5.8a), TLR-2 (Figure 5.8b), TLR-5 (Figure 5.8d) and TLR-9 (Figure 5.8e) were all significantly ($p \leq 0.05$) up-regulated, in response to 5e2 EPS, in HT29-19A cells. In contrast, a ~3-fold increase in TLR-4 mRNA (Figure 5.8c) was not significantly different from control expression, due to variance in results. HT29-19A cells were also challenged with Rosy EPS and, consequently, TLR-1 (Figure 5.8a), TLR-2 (Figure 5.8b), TLR-4 (Figure 5.8c) and TLR-5 (Figure 5.8d) were significantly ($p \leq 0.05$) up-regulated. However, TLR-9 mRNA expression was down-regulated ($p \leq 0.01$) to the extent that detectable levels were no longer discernible (Figure 5.8e).

Caco-2 cells were also challenged with 5e2 EPS and TLR-5 and TLR-9 mRNA was significantly ($p \leq 0.01$ and $p \leq 0.05$, respectively) up-regulated by ~5-fold (Figure 5.9d) and ~21-fold (Figure 5.9e), respectively. Conversely, TLR-4 mRNA was significantly ($p \leq 0.05$) down-regulated, exhibiting a 1.7-fold decrease in expression (Figure 5.9c). Moreover, no significant modification of TLR-1 and TLR-2 was observed (Figure 5.9a and b) in response to the EPS. Rosy EPS showed a similar pattern with up-regulation of TLR-5 (Figure 5.9d) and TLR-9 (Figure 5.9e) by 5.4-fold and ~28-fold, respectively. However, unlike 5e2 EPS, Rosy EPS did not provoke a down-regulation of TLR-4 mRNA, as no significant effects were seen (Figure 5.9c). Equally, no significant change was detected in the mRNA expression of both TLRs-1 and -2 (Figure 5.9a and 5.9b).

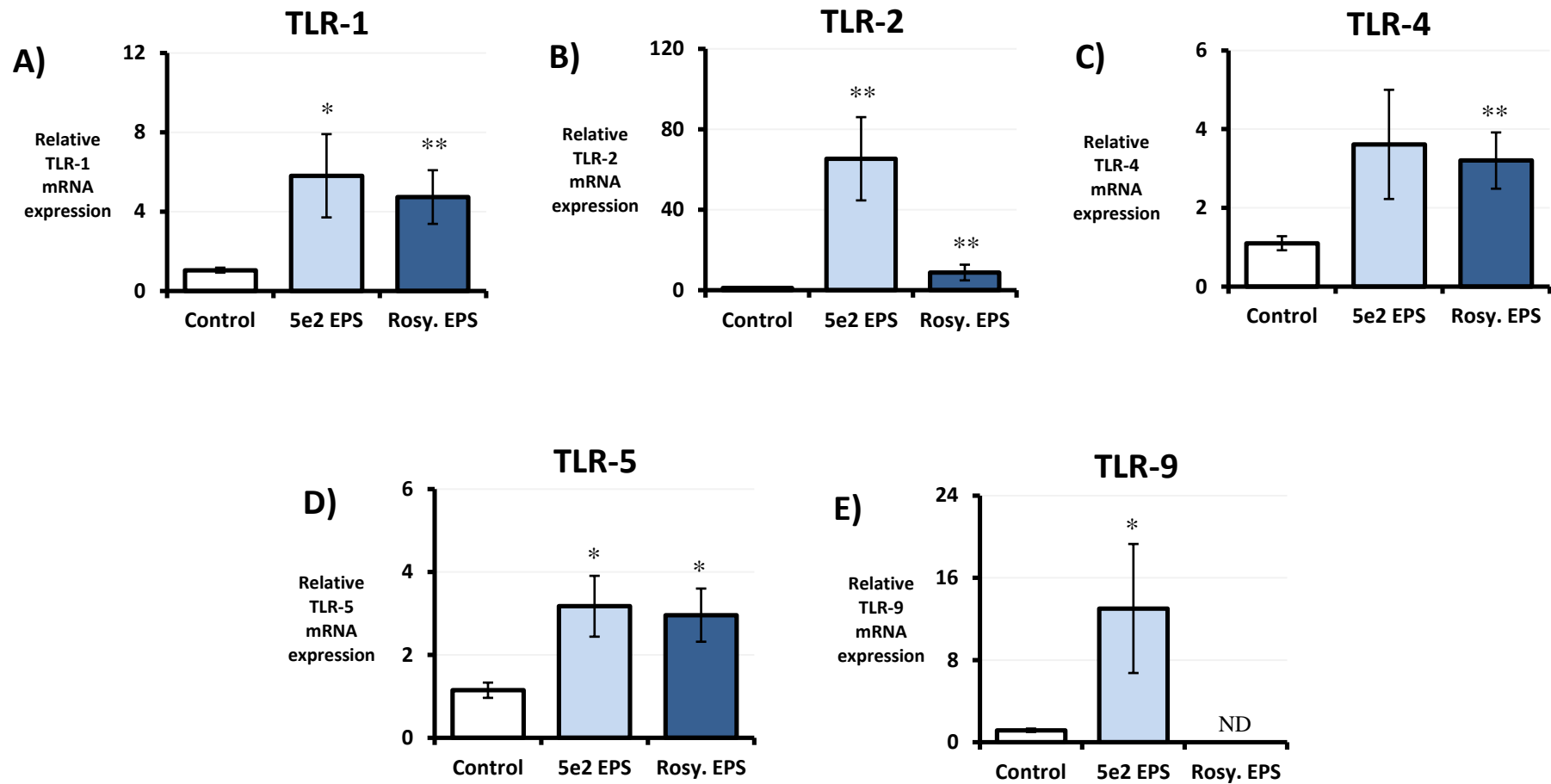


Figure 5.8 – Modification of TLR mRNA expression, in HT29-19A cells, by EPSs from *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski. HT29-19A cells were challenged with 20 µg/ml ultrapure EPSs for 24 h. mRNA levels of TLR-1 (A), TLR-2 (B), TLR-4 (C), TLR-5 (D) and TLR-9 (E) were analysed. Results are mean ± SEM, n = 3-4. ND = not detected

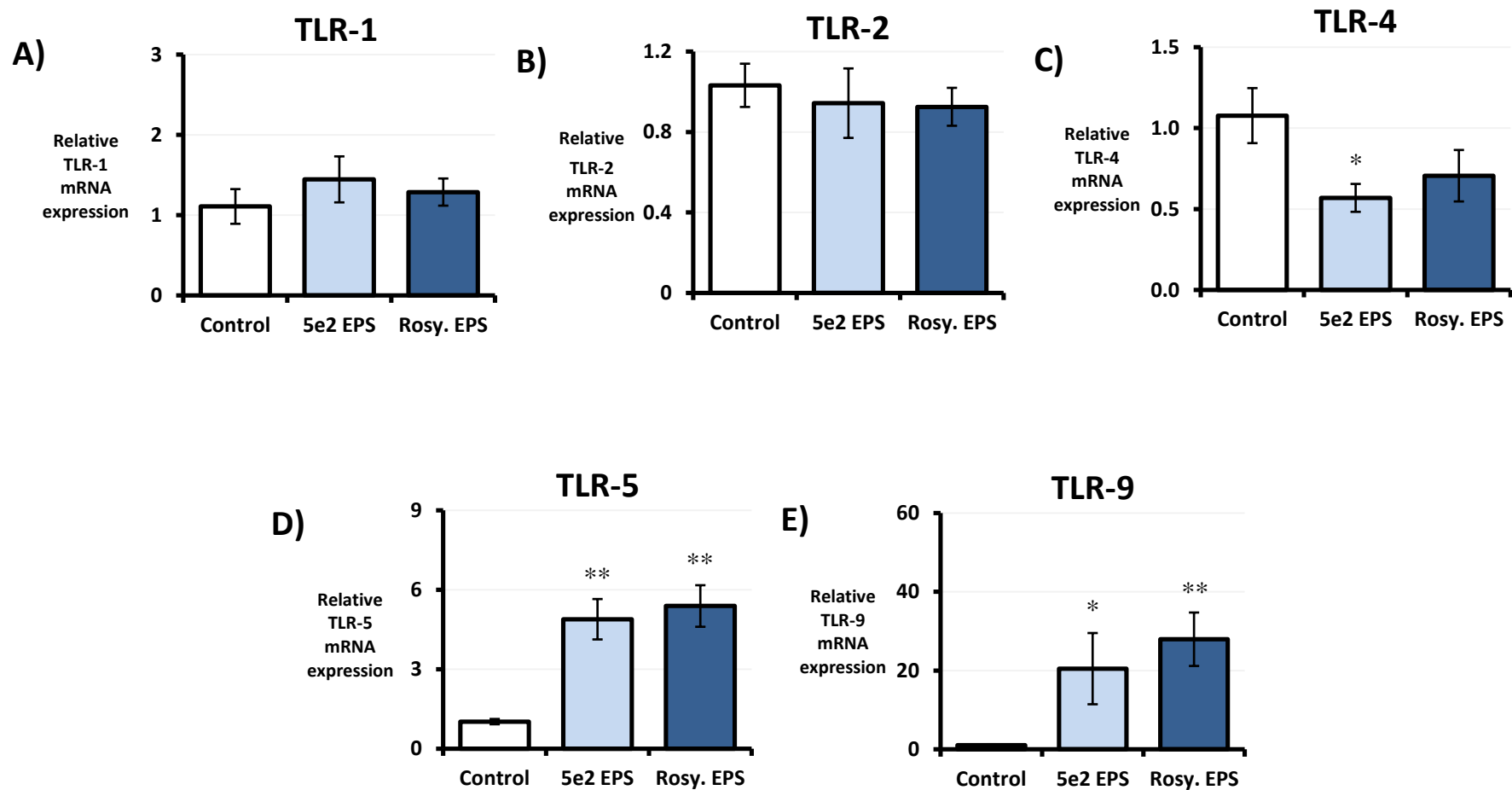


Figure 5.9 – EPSs isolated from *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski modify TLR mRNA expression in Caco-2 cells. Caco-2 cells were challenged with 20 µg/ml ultrapure EPSs for 24 h. mRNA expression of TLR-1 (A), TLR-2 (B), TLR-4 (C), TLR-5 (D) and TLR-9 (E) levels was investigated. Results are mean ± SEM, n = 3-4.

5.8 *Lactobacilli* EPS-mediated ‘priming’ of HT29-19A cells potentiates IL-8 release in response to TLR agonists

To assess whether the up-regulation of TLRs by ultrapure EPSs represented a ‘priming’ of intestinal epithelial cell lines to TLR agonists, pre-treatment with EPSs prior to challenging epithelial cells was undertaken. Cells were challenged with maximal agonist concentrations used previously in Section 3.4.

HT29-19A cells pre-treated with EPSs showed a small, but statistically significant ($p \leq 0.05$), increase in release of IL-8, in response to peptidoglycan (500 $\mu\text{g/ml}$) compared to control cells ‘pre-treated’ with cell culture media alone (Figure 5.10a). Additionally, cells pre-treated with Rosy EPS and subsequently challenged with flagellin (100 ng/ml) demonstrated a significantly augmented release of IL-8 (Figure 5.10a). Conversely, cell layers pre-treated with 5e2 EPS, followed by challenge with flagellin, showed no significant deviation from the control. Moreover, pre-treatment with EPSs did not significantly modify IL-8 release in reaction to LPS (1000 ng/ml) and ODN (50 $\mu\text{g/ml}$) (Figure 5.10a). Caco-2 cells pre-treated with EPSs did not show any significant modulation in IL-8 release in response to the subsequent challenge with flagellin or ODN (Figure 5.10b).

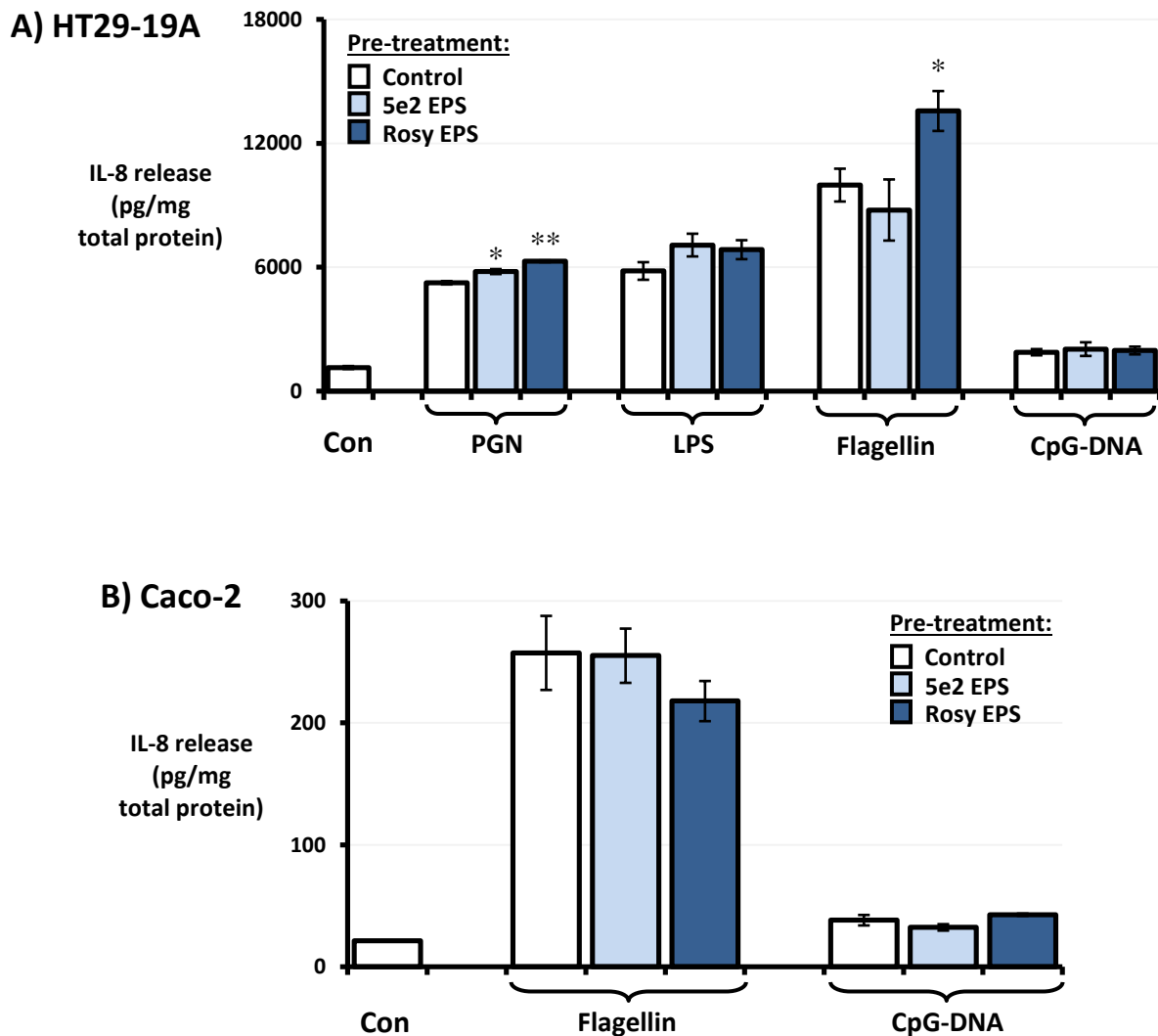


Figure 5.10 – HT29-19A cells show ‘priming’ by pre-treatment with EPSs. Cells were challenged with 20 $\mu\text{g/ml}$ EPSs for 24 h. EPS-containing media was removed and HT29-19A cells (**A**) were challenged with peptidoglycan from *B. subtilis* (500 $\mu\text{g/ml}$), LPS from *E. coli* C25 (1000 ng/ml), flagellin from *S. typhimurium* (100 ng/ml) or unmethylated CpG oligonucleotides (ODN) from *E. coli* strain B (50 $\mu\text{g/ml}$) for 24 h. Caco-2 cells (**B**) were challenged with flagellin (100 ng/ml) and ODN (50 $\mu\text{g/ml}$) for 24 h and IL-8 secretion was measured. Results are mean \pm SEM, $n = 3$.

5.9 Lactobacilli EPSs do not modify the proinflammatory effects of *C. difficile* cell-free supernatant

The potential for EPSs to modulate IL-8 release stimulated by a 1 in 10 dilution of *C. difficile* cell-free supernatant was tested, however, pre-treatment with EPSs did not significantly modulate the proinflammatory response in either HT29-19A (Figure 5.11a) or Caco-2 (Figure 5.11b) cells.

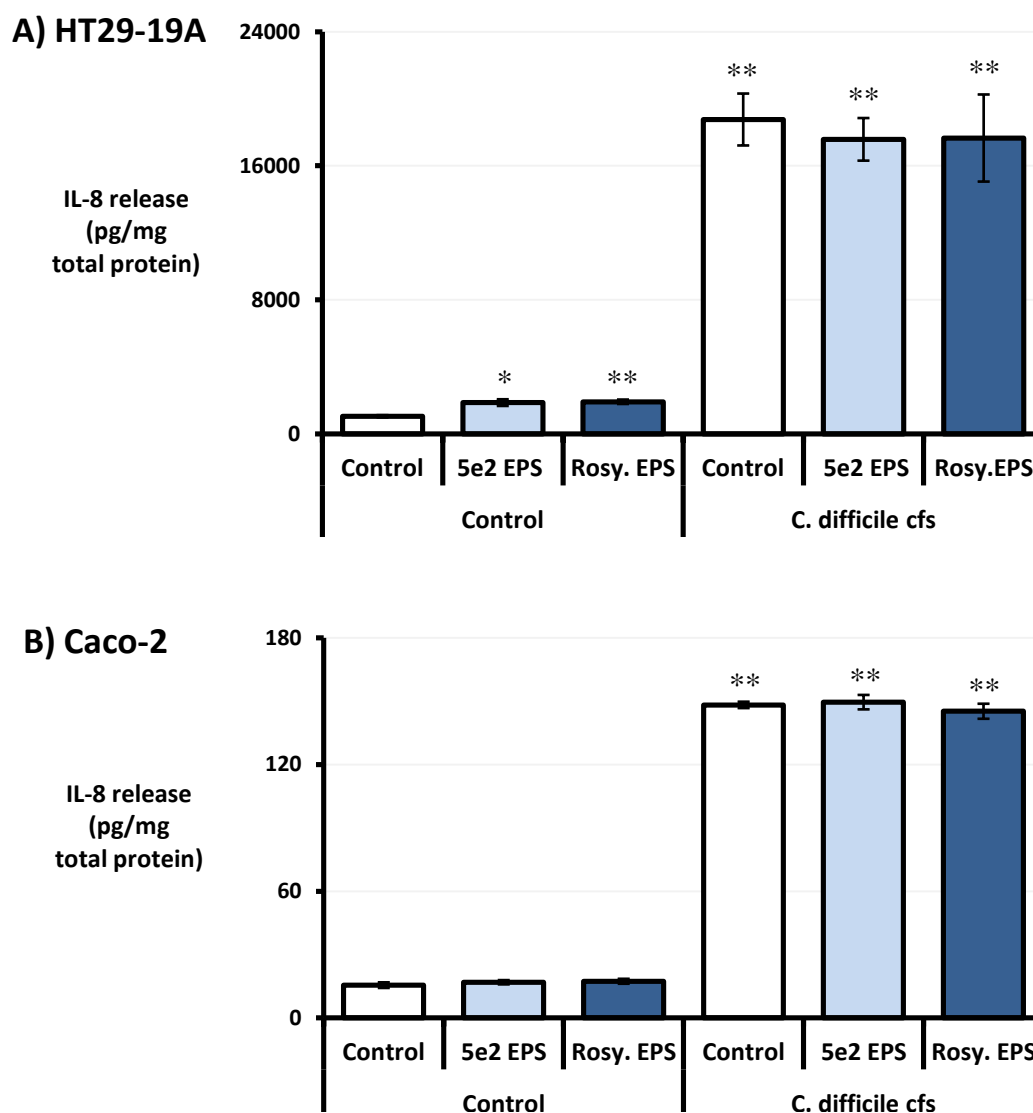


Figure 5.11 – Lactobacilli EPSs do not affect IL-8 release stimulated by *C. difficile* cell-free supernatant. HT29-19A (A) and Caco-2 (B) cells were challenged with 20 µg/ml EPSs for 24 h. Subsequently, the EPS-containing supernatant was removed, cells were challenged with a 1 in 10 dilution of *C. difficile* cfs for 24 h and IL-8 was analysed. Results are mean \pm SEM, n = 3.

5.10 *Lactobacillus* EPSs do not protect against the loss of barrier function in Caco-2 cells in response to *C. difficile* cell-free supernatant

To test if the EPSs had any effects on the tight junctions, and ultimately the barrier function, of Caco-2 cells, dome numbers were monitored during challenge experiments. 5e2 EPS did not induce any significant deviation from the number of domes seen in the unstimulated control cells over the 144 h observation period (Figure 5.12). In contrast, Rosy EPS caused an acute disruption of the barrier function immediately following the challenge, with the dome numbers significantly decreased compared to the control numbers. However, 24 h after the initial cell challenge, the dome numbers recovered to a level comparable to the control and no further deviation was seen (Figure 5.12).

The potential of EPSs in protection against decrease in barrier function, stimulated by a 1 in 100 dilution of *C. difficile* cfs, was investigated, however, neither EPSs could prevent the significant ($p \leq 0.05$) loss of domes in response to *C. difficile* cell-free supernatant (Figure 5.13).

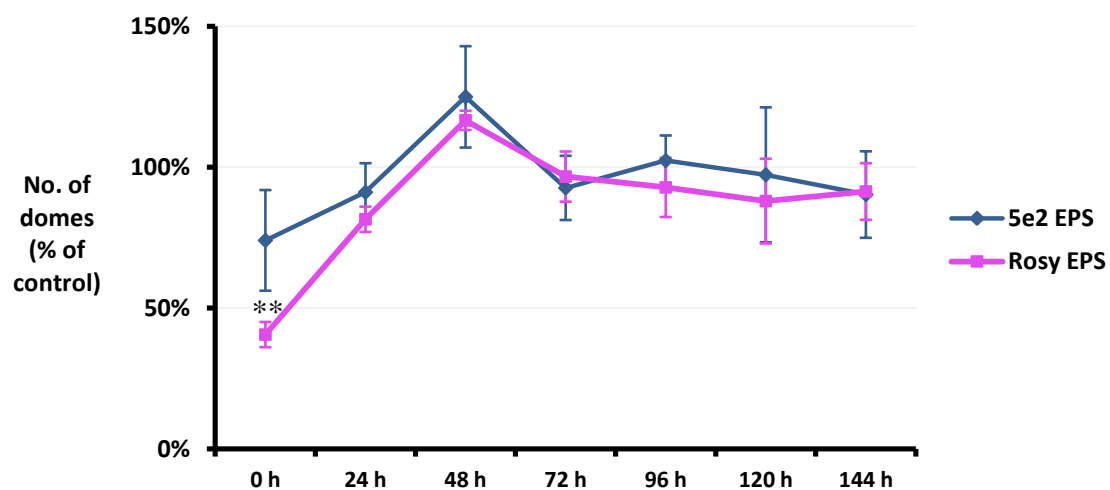


Figure 5.12 – Effect of ultrapure EPSs on barrier function of Caco-2 cells. Caco-2 cells were challenged with 20 $\mu\text{g/ml}$ of 5e2 and Rosy EPSs, for 24 h. Dome numbers were counted post-challenge and at 24 h intervals thereafter. Results were normalised to the control and expressed as a %. Results are mean \pm SEM, n = 6-9.

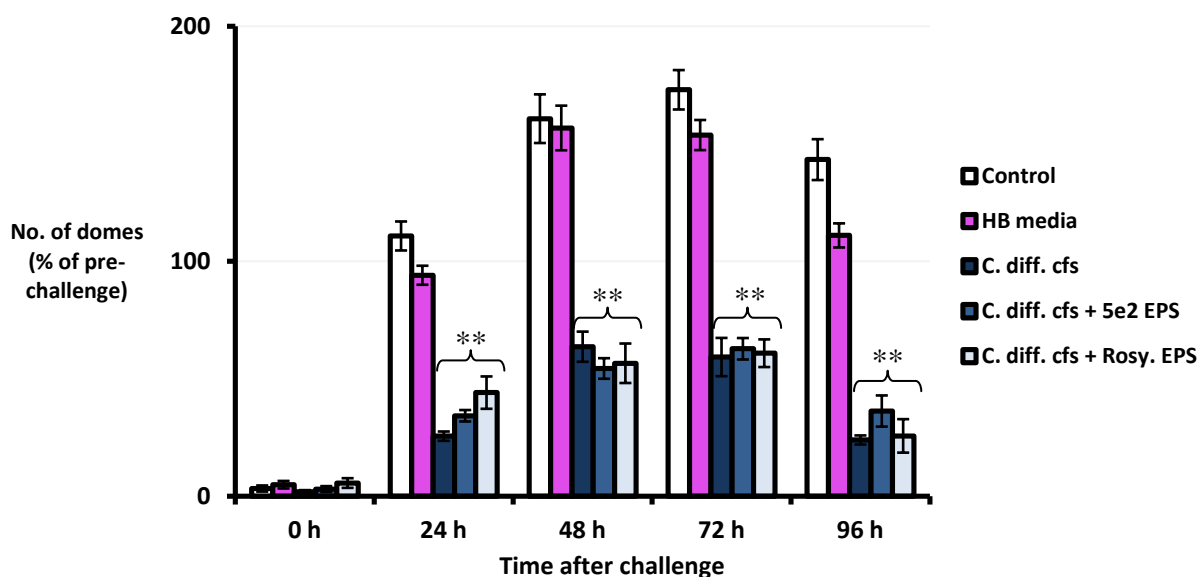


Figure 5.13 –Lactobacillus EPSs do not protect against loss of barrier integrity by *C. difficile* cell-free supernatant. Caco-2 cells were cultured until confluent and domes are apparent. The epithelial cells were pre-treated with 20 µg/ml of lactobacilli EPSs and incubated for 24 h. Cells were subsequently challenged with 1 in 100 dilutions of *C. difficile* cell-free supernatant and its horse blood media control. Results are expressed as a % of number of domes observed prior to pre-treatments and cell challenges. Results shown are mean, n = 6. ** indicates significance from 100 %.

5.11 *L. helveticus* sp. Rosyjski EPS modulates bacterial adherence and internalisation in IECs

The modulatory effects of EPSs on bacterial adherence and internalisation were explored. Pre-treatment of HT29-19A and Caco-2 cell lines with 5e2 EPSs had no significant effect on the adherence (Figure 5.14a) or internalisation (Figure 5.14b) of *E. coli* C25. Similarly, pre-treatment of HT29-19A cells with Rosy EPS resulted in little deviation from the basal level of bacterial adherence (Figure 5.14a), however, the number of bacteria undergoing internalisation was significantly ($p \leq 0.01$) increased (Figure 5.14b). Conversely, Caco-2 cells pre-treated with Rosy EPS demonstrated a significant increase in bacterial adherence (Figure 5.14a), but did not exhibit modulation of bacterial internalisation (Figure 5.14b).

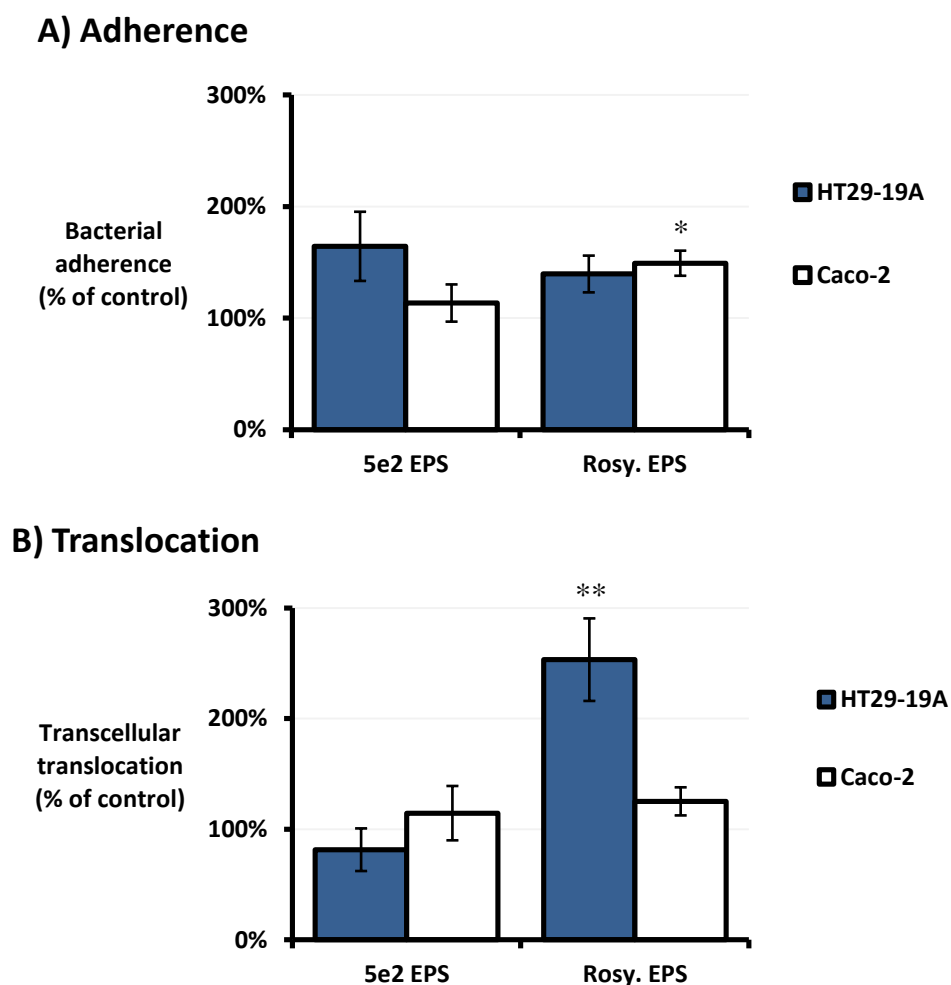


Figure 5.14 – *L. helveticus* sp. Rosyjski EPS significantly increases bacterial adherence in Caco-2 cells and bacterial translocation in HT29-19A cells. Epithelial cells were challenged with 20 µg/ml EPSs for 24 h. Subsequently, the cells were co-cultured with $\sim 1 \times 10^9$ CFU *E. coli* C25 for 4 h. Adherent and internalised bacteria (**A**) were plated out and counted. Alternatively, adherent bacteria were killed and bacteria inside epithelial cells (**B**) were plated out and counted. Results were expressed as a % of the original bacterial inoculum and were normalised to the control. Results are mean \pm SEM, n = 6-8.

5.12 *Lactobacilli* EPSs do not modulate growth of *E. coli* C25

Incubation of *E. coli* C25 in Trans-HBSS gave a ~1.5-fold increase in the number of bacteria compared to the original inoculum and the presence of EPSs failed to significantly modify this figure (Figure 5.15).

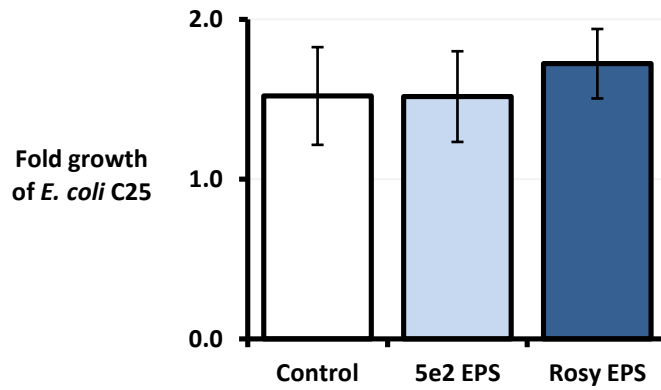


Figure 5.15 – Lactobacilli EPSs do not significantly modulate the growth of *E. coli* C25.

1×10^9 CFU *E. coli* C25 were incubated for 4 h, in the presence of 20 $\mu\text{g/ml}$ EPSs. Subsequently, cultures were serially diluted and plated out in TSA. Plates were incubated overnight and the resultant colonies were counted. The post-incubation colony count is expressed as a fold change in bacteria, compared to the original inoculum. Results are mean \pm SEM, n = 4-6.

5.13 Summary

This chapter investigated the immunomodulatory activity of extracellular products from two uncharacterised EPS-producing lactobacilli strains, *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski.

HT29-19A cells appeared largely unreactive to cell-free supernatants and sonicates from both strains of lactobacilli, with only a minimal, yet statistically significant, increase in IL-8 expression exhibited in response to a 1 in 10 dilution of 5e2 cell-free supernatant. Nevertheless, cell-free supernatants from both strains of lactobacilli were found to differentially regulate the mRNA expression of TLRs. Caco-2 cells were more responsive to the two strains of lactobacilli, exhibiting significant potentiation of IL-8 in response to 1 in 10 dilutions of cell-free supernatants and sonicates. However, cell-free supernatants had little effect on TLR expression in Caco-2 cells, with only TLR-9 down-regulated by 5e2 cfs. Conversely, sonicates from both strains of lactobacilli showed significant modulatory activity on the expression of TLR-1, TLR-2 and TLR-9. Cell-free supernatants from both lactobacilli strains were unable to stimulate expression or release of IL-10 in either cell line, but 5e2 cfs was seen to actively down-regulate transcriptional expression of the anti-inflammatory mediator in Caco-2 cells.

Extracellular products from both strains of lactobacilli were unable to modulate the IL-8 release stimulated by *C. difficile* cell-free supernatant.

Both 5e2 and Rosy EPSs demonstrated proinflammatory activity in the HT29-19A cell line, provoking significant release and expression of IL-8. However, in Caco-2 cells, only 5e2 EPS was able to stimulate a small, but significant up-regulation in IL-8 mRNA. Additionally, EPSs exhibited significant modulation of TLR expression in both HT29-19A and Caco-2 cell lines. In contrast, neither EPS was able to modulate the expression, or release, of IL-10.

Pre-treatment of HT29-19A cells with EPSs resulted in the potentiation of IL-8 release in response to both peptidoglycan and flagellin, however, IL-8 release in response to lipopolysaccharide and CpG DNA was unaffected. Caco-2 cells pre-treated with EPSs exhibited no significant modulation of IL-8 secretion in response to either flagellin or CpG DNA. Moreover, neither EPS was able to regulate IL-8 secretion in either cell line, or the decrease of epithelial barrier function in Caco-2 cells, as stimulated by *C. difficile* cell-free supernatant.

Pre-treatment with Rosy EPS significantly increased bacterial adherence in Caco-2 cells and internalisation of *E. coli* C25 in the HT29-19A cell line, however, neither of these effects were due enhancement of bacterial proliferation.

Chapter 6

Discussion

6.1 Phenotypic differences in the HT29-19A and Caco-2 cell lines

The effectiveness of *in vitro* cell models is limited by the loss of important anatomical and biochemical features of the *in vivo* organ; therefore, extrapolating data back to an *in vivo* system is very difficult (LeFerrec *et al.*, 2001). However, *in vitro* models can provide vital insights in to metabolism and immunity at a cellular level and are thus used extensively in the preliminary stages of drug development (Alley *et al.*, 1988; Artursson *et al.*, 2001). The rationale for utilising the HT29-19A and Caco-2 *in vitro* human intestinal epithelial cell lines in this investigation was their previous use in modelling bacterial interactions with the intestinal epithelium (Coconnier *et al.*, 1993; Coconnier *et al.*, 1997; Tuomola and Salminen, 1998; Lammers *et al.*, 2002; Bannon *et al.*, 2009).

The phenotypic differences and basic properties between the two immortalised intestinal epithelial cell lines HT29-19A and Caco-2 were investigated, with the aim of selecting the most appropriate with which to continue. Expression of Toll-like receptors (TLRs), a major group of pattern recognition receptors (PRRs) found in intestinal epithelial cells, and the inflammatory cytokines interleukin (IL)-8 and IL-10 were studied. Additionally, the ability of the cell lines to form tight junctions, and thus form an epithelial barrier, was tested (discussed in Section 6.3.2).

6.1.1 Toll-like receptors

TLRs are a group of membrane glycoproteins which recognise and bind a wide range of highly conserved microbial-associated molecular patterns (MAMPs) and mediate an immune reaction in response to binding of their respective ligand(s) (Medzhitov, 2001). Qualitative analysis of TLR expression, via reverse transcription (RT)-PCR, has previously been undertaken with the HT29-19A and Caco-2 cell lines (Bannon, 2008). This preliminary work has been expanded upon in this study, with differences in expression measured utilising

quantitative real-time (qRT)-PCR. TLR mRNA levels were calculated relative to transcriptional expression of housekeeping genes (genes which are expressed in all nucleated cells of the body and allow direct comparison between different cell types), in this case, β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In concordance with the qualitative comparison undertaken by Bannon (2008), HT29-19A and Caco-2 cells demonstrated transcriptional expression of all TLRs tested (TLR-1, TLR-2, TLR-4, TLR-5 and TLR-9), suggesting both cell lines possessed the capability to produce receptors which respond to a wide variety of known bacterial antigens. The two cell lines exhibited little variation in their expression of TLR-1, TLR-5 and TLR-9 mRNA, relative to the housekeeping genes; however, they demonstrated marked differences in the mRNA expression of TLR-2 and TLR-4. HT29-19A cells were shown to express ~700 times less TLR-2 mRNA than Caco-2 cells, thus suggesting significantly less potential for responsiveness to TLR-2 agonists, such as peptidoglycan (PGN) and lipoprotein (LP), in comparison to the Caco-2 cell line. In contrast to this, the HT29-19A cell line exhibited ~340 times higher expression of TLR-4 mRNA than Caco-2 cells, suggestive of potential for an increased reactivity to lipopolysaccharide (LPS), compared to Caco-2 cells. Should this be the case in response to the bacterial agonists, then the two cell lines would effectively be reactive to bacterial products that the other is not, thus covering the range between them.

6.1.2 Inflammatory cytokines

In this study, the capacity for the HT29-19A and Caco-2 cell lines to spontaneously produce inflammatory cytokines was investigated, with the expression and release of the major proinflammatory mediator IL-8 and the anti-inflammatory immunoregulator IL-10 compared between the two cell lines.

IL-8 is commonly used in stimulatory studies of intestinal epithelial cell lines with bacteria (Eckmann *et al.*, 1993b; Jung *et al.*, 1995) and bacterial products (Schuerer-Maly *et al.*, 1994; Streiner *et al.*, 2000; Akhtar *et al.*, 2003), as it plays a key role in the initiation of the innate immune system in both health and disease (Baggiolini *et al.*, 1989; Harada *et al.*, 1994; Daig *et al.*, 1996). Previous studies have demonstrated that the HT29-19A and Caco-2 cell lines possess different capacities for IL-8 release, with HT29-19A able to secrete much higher levels than Caco-2 cells (Warhurst *et al.*, 1998; Bannon, 2008).

In the present investigation, and in accordance with previous studies, HT29-19A cells were shown to express significantly higher levels of IL-8 than Caco-2 cells. At maximal release, HT29-19A cells secreted ~64 times more IL-8 than Caco-2 cells and this was paralleled at the transcriptional level, with HT29-19A cells expressing ~74-fold more IL-8 mRNA than Caco-2 cells. It is thought that the variation in IL-8 release between the two cell lines is likely to reflect phenotypic differences, rather than other factors, such as level of differentiation of the cell layers (Warhurst *et al.*, 1998). It has been speculated that the HT29 and Caco-2 cell lines were isolated from populations of different cell types within the *in vivo* epithelial layer, with HT29 cell lines thought to originate from intestinal epithelial crypt cells (Huet *et al.*, 1987; Velcich *et al.*, 1995; Warhurst *et al.*, 1998), whereas Caco-2 cells were potentially derived from absorptive villus enterocytes (Delie and Rubas, 1997; Yee, 1997; Warhurst *et al.*, 1998). *In vivo*, crypts exist in a state of sterility to protect the highly vulnerable epithelial stem cells (Porter *et al.*, 2002; Elphick and Mahida, 2005); therefore, the high basal release of the potent proinflammatory chemokine IL-8 in HT29-19A cells is potentially representative of highly primed intestinal crypt cells, *in vivo*. Conversely, Caco-2 cells exhibit a much lower capacity for constitutive IL-8 release. Low basal IL-8 production is potentially a trait typical of absorptive villus enterocytes, which must remain relatively hyporesponsive, thus preventing excessive inflammation and the pathogenesis of chronic

diseases, such as IBD (Otte *et al.*, 2003).

IL-10 is a key immunoregulator in intestinal homeostasis, with its absence heavily linked with the pathogenesis of IBD (Steidler *et al.*, 2000; Mosser and Zhang, 2008; de Moreno de LeBlanc *et al.*, 2011) and it also plays an important role in the suppression of excessive inflammation during infection (Couper *et al.*, 2008). Expression of IL-10 is rarely considered in non-immune cells, such as epithelial cells, however, both the HT29 and Caco-2 cells lines have been shown to express IL-10 at the transcriptional level (Bahrami *et al.*, 2010). Additionally, a recent study by Gao *et al.* (2012) demonstrated novel expression and release of IL-10 in HT29 cells, in response to challenge with *Clostridium butyricum*, thus demonstrating the capacity of these cells to produce the immunosuppressor. Nevertheless, HT29-19A subclone cells, used in this investigation, appeared unable to spontaneously produce IL-10, as no detectable levels, or indeed, transcriptional expression, was observed. In contrast, Caco-2 cells exhibited a discernible mRNA signal, thus suggesting that IL-10 is potentially inducible in these cells; however, no detectable levels of IL-10 protein were found. It is possible that any protein produced was rapidly degraded in solution, however, IL-10 is relatively stable (Kenis *et al.*, 2002), therefore, this is unlikely. An alternative explanation is that, in Caco-2 cells, IL-10 mRNA could be present in a state of quiescence within processing (P) bodies (Parker and Sheth, 2007). P bodies are aggregates of untranslating mRNAs (Texeira *et al.*, 2005) and proteins involved in mRNA repression and degradation (Ingelfinger *et al.*, 2002, Cougot *et al.*, 2004), in which mRNAs can be sequestered until required for translational expression (Parker and Sheth, 2007). Furthermore, it is possible that the primers are not specific to the *IL-10* gene and are falsely causing positive results in qRT-PCR. Genetic sequencing of the primers would determine whether they were in fact specific to the *IL-10* gene.

6.2 Intestinal epithelial cell responses to pathogenic TLR agonists

TLR-mediated immunity in the intestinal epithelial cell lines HT29, Caco-2 and T84 is relatively well characterised, with previous studies documenting IL-8 release in response to pathogenic bacterial products, such as flagellin (Streiner *et al.*, 2000; Gewirtz *et al.*, 2001), LPS (Schuerer-Maly *et al.*, 1994) and CpG DNA (Ewaschuk *et al.*, 2007). This study utilised commercially-sourced bacterial TLR agonists in order to investigate changes in expression of TLRs and IL-8 in HT29-19A and Caco-2 cells, in response to stimulation. A 24 h incubation period was utilised in an attempt to mimic the chronic exposure to these agonists in the *in vivo* intestine.

Consequently, the results of this investigation showed that HT29-19A cells were responsive to the range of agonists tested, with significant augmentation of IL-8 observed at both the transcriptional and translational levels. The Caco-2 cell line also showed responsiveness to the agonists with the exception of LPS, which, in accordance with a study by Eckmann *et al.* (1993a), did not provoke any significant increase in IL-8 release. However, quite curiously, the lack of IL-8 release was contrasted at the transcriptional level, with a significant increase in IL-8 mRNA expression observed in response to LPS. This phenomenon could be due to LPS-mediated inhibition of IL-8 mRNA translation, with the extra copies of IL-8 mRNA possibly being sequestered in the aforementioned P bodies. Additionally, having shown earlier that constitutive release of IL-8 was significantly higher in HT29-19A cells; it was unsurprising that they also showed a greater capacity for maximal stimulated release, compared to the Caco-2 cell line.

As hypothesised earlier, the mRNA expression of TLRs in the two cell lines appeared to have a profound influence on their sensitivity to the bacterial antigens tested. Having shown similar expression levels of TLR-5 and TLR-9 mRNA, both cell lines demonstrated comparable sensitivities to both flagellin and CpG DNA, respectively. However, Caco-2

cells, which were found to express ~700 times more TLR-2 mRNA than the HT29-19A cells (Section 3.2), were sensitive to 100-fold lower concentrations of PGN; nevertheless, HT29-19A cells did still exhibit a moderate degree of responsiveness to PGN. Interestingly, previous studies have shown PGN has no stimulatory effects on HT29 and Caco-2 cell lines (Melmed *et al.*, 2003; Furrie *et al.*, 2005); however, this discrepancy could be due to differences in protocol, as the previous studies employed only 3 h (Furrie *et al.*, 2005) and 5 h (Melmed *et al.*, 2003) cell challenges, respectively, while the present study utilised a 24 h exposure. In contrast to the sensitivity to PGN, HT29-19A cells were >100-fold more sensitive to LPS than Caco-2 cells, a trait consistent with their ~340 times higher expression of TLR-4 mRNA. Indeed, Caco-2 cells appeared totally unresponsive to the range of LPS concentrations tested, with no significant changes in IL-8 release observed. These findings suggested that it was beneficial to use both cell lines as they possess different, but complementary, phenotypes, which are, in all probability, reflective of their respective origins.

The expression of TLRs is not static, but highly modulated in response to various stimuli, such as cytokines and environmental stresses (Akira *et al.*, 2006) and previous studies have reported that antigen binding to professional immune cells results in the up-regulation of their cognate TLR receptors (Nomura *et al.*, 2000; Visintin *et al.*, 2001; Hornung *et al.*, 2002). The present investigation sought to investigate if this phenomenon also occurred in the non-immune intestinal epithelial cells. Consequently, this is the first study to show a wide range of TLR agonists stimulating an up-regulation of their respective cognate receptor(s) in intestinal epithelial cells. However, in reaction to CpG DNA (50 µg/ml), a complete down-regulation of TLR-9 mRNA was observed in HT29-19A cells, with no detectable levels present, a result which contrasts the significant up-regulation in TLR-9 expression observed in a study by Ewaschuk *et al.* (2007). Nevertheless, these data suggested

desensitisation, or tolerance, of the epithelial cell line to subsequent challenge with TLR-9 agonists, a homeostatic phenomenon previously described with HCA-7 intestinal epithelial cells, in a key study by Lee *et al.* (2006). This effect could be important in an *in vivo* system, as it would potentially act to prevent excessive inflammation in response to high concentrations of CpG DNA in the intestinal lumen. Additionally, in response to LPS, Caco-2 exhibited a small, but statistically significant, transcriptional down-regulation of the LPS-binding co-factor, CD14.

In this investigation, the expression of TLRs was studied semi-quantitatively, with relative fold differences between the two cell lines calculated; however, fully quantitative comparisons could have been made by calculating the concentration of TLR mRNA present in the cells. This could be achieved by comparing the resultant *Ct* value from the target sample to a standard curve created from serial dilutions of a standard of known concentration (giving concentration vs. *Ct* value). Additionally, TLR expression was explored exclusively at the transcriptional level, and, given that transcriptional expression has a relatively weak correlation ($r=0.4$) to translational expression (Schwanhäusser *et al.*, 2011), the level of mRNA is not necessarily directly proportional to the amount of protein expressed. Therefore, although the variation in TLR mRNA expression reported in this investigation was, to an extent, representative of differences in functionality, protein quantification (via Western blot analysis or flow cytometry) is required for a more definitive change in TLR expression in response to the different bacterial agonists. Furthermore, given that TLRs signal through a common intracellular pathway (Aderem and Ulevitch, 2000; Akira *et al.*, 2006), investigation of expression changes in non-cognate TLR receptors (via a feedback mechanism hypothesised by Viszoso Pinto *et al.*, (2009)) in response to the various bacterial agonists would be an interesting development on the current study.

6.3 Activity of *C. difficile* cell-free supernatant on intestinal epithelial cells

6.3.1 Immunostimulatory activity

Previous studies investigating the immunomodulatory activity of *C. difficile* extracellular products, on HT29 and T84 intestinal epithelial cell lines, have generally utilised purified toxins (TcdA and TcB) (Feltis *et al.*, 2000; Nusrat *et al.*, 2001; Lica *et al.*, 2011). The host cell surface receptor(s) that confer responsiveness to these toxins are currently unknown; however, there is evidence to suggest that they are non-proteinaceous, but likely to be carbohydrate-based moieties (Krivan *et al.*, 1986; Tucker and Wilkins, 1991). Therefore, it can be speculated that any immunological activity will be mediated via a TLR-independent pathway.

In the present study, *C. difficile* crude cell-free supernatant (cfs) was utilised to give a more accurate representation of the full range of extracellular products secreted by the enteropathogen *in vivo*, which include, but are not limited to, the toxins, TcdA, TcdB and CDT. Potential stimulatory effects of the untreated horse blood broth were investigated; however, no significant changes in IL-8 release were observed, thus facilitating this work. Both HT29-19A and Caco-2 cell lines showed sensitivity to the inflammatory effects of *C. difficile* cfs, with significant increases in both transcriptional and translational expression of IL-8 induced. These results concur with those gained in a study by Canny *et al.* (2006), which showed *C. difficile* crude cell-free supernatant (cfs) elicited IL-8 from HT29 and T84 intestinal epithelial cell lines. Additionally, IL-8 release by the HT29-19A cell line was much higher than that observed in Caco-2 cells; however, the magnitude of augmentation was comparable in the two cell lines, relative to their respective basal levels. Nevertheless, HT29-19A cells did appear more sensitive to the inflammatory effects of *C. difficile* cfs, with more dilute samples stimulating significant IL-8 release than in Caco-2 cells. This was possibly related to the difference in origins of the two cell lines, as; *in vivo*, sterile crypt cells (HT29-

19A cells) would likely be much more responsive to the presence of products from *C. difficile* than enterocytes (Caco-2), which are continuously exposed to the microfloral milieu.

6.3.2 Disruption of barrier function of Caco-2 cells

Generally, the ability of *in vitro* cell lines to form tight junctions, and the consequential integrity of cell layers, is tested by monitoring the trans-epithelial electrical resistance (TEER) during cell challenges (Canil *et al.*, 1993; Balda *et al.*, 1996). However, since the facilities to perform such experimentation were not available in this study, an alternative was sought. A number of *in vitro* cell lines are able to polarise to such an extent that the unidirectional flow of ions and water molecules, from the culture media, results in an accumulation of fluid between the cell monolayer and surface of the culture vessel (Toyoshima *et al.*, 1976; Rabito *et al.*, 1980; Su *et al.*, 2007). Consequently, local elevation of cells, from the geometric plane of the culture vessel, occurs and a multicellular hemicyst, or dome, forms (Figure 2.1). The presence of domes requires physiologically intact intercellular tight junctions; therefore, the maintenance of these structures can be indicative of the monolayer's integrity. HT29-19A cells were not able to form domes; conversely, once confluence had been reached, Caco-2 cells formed significant numbers of domes, and maintained them for up to 14 days subsequent to this. These results emphasise the increased ability of Caco-2 cells to form tight junctions, compared to HT29-19A cells.

In response to higher concentrations (neat, 1 in 10 and 1 in 100) of *C. difficile* cell-free supernatant, the number of domes present on Caco-2 cell monolayers was significantly, and irreversibly, diminished. These results suggest that barrier integrity of the monolayers was significantly decreased, a result which is consistent with the data gained by Sutton *et al.* (2008), who showed that *C. difficile* cell-free supernatant is capable of decreasing TEER in Caco-2 cells. The decrease in barrier integrity is due to morphological changes in the

epithelial cells in response to the toxins released by *C. difficile*. Cell rounding, in response to purified *C. difficile* toxins, was first observed in human amnion cells by Chang *et al.* (1979) and have since been described in human intestinal epithelial cell lines (HT29, Caco-2 and T84 cells) and in primary colon epithelial cells (Mahida *et al.*, 1996). *C. difficile* toxins cause degradation of filamentous (F-)actin to monomeric (G-)actin (Carter *et al.*, 2011), via the inactivation of Rho GTPases, the molecular switches which normally control the regulation of the F-actin cytoskeleton within epithelial cells (Davies *et al.*, 2011). Degradation of the F-actin cytoskeleton ultimately results in rounding of the cells, loosening of the intercellular tight junctions and an overall decrease in the integrity of monolayers of intestinal epithelial cells (Nusrat *et al.*, 2001; Berkes *et al.*, 2003; Zemljic *et al.*, 2010).

6.4 Immunomodulatory activity of *E. coli* C25 extracellular products

A chronic low level of inflammation, induced by the commensal microflora, is thought to be characteristic of intestinal homeostasis, thus ‘priming’ the intestinal immunity for a more efficient inflammatory response to the presence of pathogens (Macpherson and Harris, 2004; Artis, 2008). However, the significance of microfloral extracellular products in this process has not previously been explored. As one of the first species of bacteria to colonise the human neonatal intestine (Hooper, 2004), it can be hypothesised that *E. coli* potentially play a key role in the establishment of the homeostatic equilibrium within the intestine. The immunostimulatory ability of cell-free supernatant (cfs) and sonicates from the enteric commensal bacterium, *E. coli* C25, have previously been considered by Bannon (2008), who showed significant IL-8 release elicited from HT29-19A cells, in a 4 h challenge experiment. This study sought to further explore the inflammatory potential of C25 extracellular products, and utilised a much extended (24 h) exposure protocol.

In concordance with the results gained by Bannon (2008), C25 cfs provoked a significant increase in IL-8 release in HT29-19A cells. This was also paralleled at the transcriptional level, with a significant increase in IL-8 mRNA expression. A similar pattern was also observed in Caco-2 cells, with both transcriptional and translational expression elicited in response to C25 cfs. Also, an mRNA up-regulation of the anti-inflammatory cytokine, IL-10, was observed in both cell lines, in response to C25 cfs, a result particularly significant in HT29-19A cells, since no constitutive expression was previously detected. These data were potentially suggestive of a role in the homeostatic balance of inflammation and its suppression, in the intestine, for the commensal bacterium, however, stimulated levels of IL-10 transcription were still representative of relatively low levels of mRNA and, consequently, the protein was not detected. C25 cfs also showed modulatory effects on the mRNA expression of TLR-1, TLR-4 and TLR-9, stimulating significant increases of all three

receptors. The modulation of TLR-1 and TLR-4 mRNA is consistent with earlier data which indicated that binding of their respective agonists causes their up-regulation; however, the increase in TLR-9 mRNA contradicts the data obtained in response to pathogenic CpG-DNA (see Section 3.4). It is possible that CpG content differences between the pathogenic and C25 CpG-DNA have different modulatory effects on TLR-9 expression, however, it is more likely that the up-regulation is caused indirectly, via stimulation by LPS, a phenomenon previously described in murine macrophages (An *et al.*, 2002).

Subsequently, ultrasonic disruption of bacteria was utilised in an attempt to increase the release of bacterial cellular components, thus going some way to mimicking the increased lysis of bacteria in the intestinal lumen, in response to the host immunity. In both HT29-19A and Caco-2 cells, the proinflammatory IL-8 response to challenge with C25 sonicate showed a similar pattern to C25 cfs challenge, with a significant up-regulation of both expression and release observed. However, the stimulatory effects of C25 sonicate were greater than the cfs, suggesting ultrasonic disruption did in fact increase the concentration of antigenic material in solution. Additionally, mRNA expression of TLR-2 and TLR-4 was up-regulated to a greater degree, in both cell lines, when compared to the effects of C25 cfs, thus indicating a greater presence of TLR-2 agonists, such as PGN and LP, and the TLR-4 agonist, LPS. However, a key modulatory difference between C25 sonicates and C25 cfs was the complete down-regulation of TLR-9 mRNA, in both cell lines, in response to challenge with the C25 sonicate. This effect was potentially attributable to the increased concentrations of C25 CpG-DNA in solution binding TLR-9 and subsequently down-regulating the receptor's transcriptional expression, as occurred in HT29-19A cells, in response to pathogenic CpG-DNA earlier in the investigation (Section 3.4). Attempts were made to extract CpG-DNA from cultures of C25, to confirm this effect; however, sufficient concentrations for cell challenge experimentation could not be isolated.

Live C25 has previously been shown to stimulate the proinflammatory cytokine TNF- α in both monoassociated rat models (*in vivo*) and in the Caco-2 cell line (*in vitro*), (Michalsky *et al.*, 1997). Additionally, C25 has been shown to exhibit pathogenic tendencies, such as loosening of tight junctions in T84 intestinal epithelial cells (Zareie *et al.*, 2005). However, when the inflammatory effects of C25 extracellular products, observed in this investigation, were compared with the effects in response to cell-free supernatant from the enteropathogen, *C. difficile* (Figure 6.1), it was evident that the stimulation of IL-8 release was relatively low. Therefore, it was hypothesised that this low-level inflammatory response could potentially prime the intestinal epithelial layer to pathogenic attack, giving a larger, more effective immune response. To test the hypothesis, HT29-19A and Caco-2 cells were pre-treated with C25 cfs and sonicate and subsequently challenged with *C. difficile* cfs. However, the results indicated this was not the case, as no significant differences in IL-8 release were observed in response to the pre-treatments.

6.4.1 Modulation of bacterial translocation

Translocation across the intestinal epithelium is often associated with pathogenic bacteria (Balzan *et al.*, 2007); however, it has previously been theorised that a constitutive level of translocation of commensal bacteria, via the transcellular pathway (thus leaving the epithelium intact), is involved in mucosal immune development (Berg, 1995). *E. coli* C25 has previously been used a model organism for bacterial translocation (Deitch *et al.*, 1991; Cruz *et al.*, 1994; Michalsky *et al.*, 1997; Mattar *et al.*, 2001) and it was this bacterium's capacity for a relatively low level of translocation, via the transcellular pathway, that was utilised here.

In control conditions, C25 adhered to, and was internalised by, both HT29-19A and Caco-2 cell lines, in an inoculum density-dependent manner, i.e. the number of adherent and internalised bacteria increased directly proportional to the increase of inoculum density.

However, when the numbers of adherent and internalised bacteria were expressed as a percentage of the inoculum population, there was little deviation between the range of bacterial densities tested. Therefore, C25 did not appear to acutely promote its own adherence or internalisation. However, in the protocol utilised here, the bacteria are resuspended in fresh Trans-HBSS solution prior to co-incubation with epithelial cell layers; therefore, the exposure of the cell layers to C25-derived extracellular products is likely to be quite low. Consequently, it was decided upon to investigate the effects of pre-treating the epithelial cells with C25 cfs and sonicate prior to the translocation assay.

Previously, it has been shown that pre-treatment of Caco-2 cells, with high levels of proinflammatory cytokines, such as IFN- γ and TNF- α , can increase the translocation of C25 by ~100-fold (Clark *et al.*, 2003; Clark *et al.*, 2005; Macutkiewicz *et al.*, 2008). The effects observed in this investigation were much more modest, with ~2-fold and ~4-fold increases in bacteria translocation, in response to pre-treatment of Caco-2 cells with C25 cfs and sonicates, respectively. Additionally, a ~2-fold increase in translocation was observed, in HT29-19A cells, in response to pre-treatment with C25 cfs. The low magnitude of internalisation modification, observed in this investigation, is possibly due to the much lower proinflammatory potential of the C25-derived extracellular products, compared to the cytokines used in previous studies. Nevertheless, the data obtained here does show the potential for commensal-derived products to promote the translocation of the parent bacterium across the epithelial layer. Should these effects also be seen *in vivo*, they could represent a mechanism for the development of immune tolerance to the commensal flora (Mowat, 2003). Moreover, the promotion of bacterial internalisation by commensal-derived products could also contribute to antibiotic-associated diarrhoea (Bartlett, 2002) induced by bacteriolytic antibiotics, such as ampicillin, amoxicillin and cephalosporins.

6.5 Interactions of commensal-derived MVs and intestinal epithelial cells

6.5.1 Immunomodulatory activity

As spheres of outer membrane from Gram-negative bacteria, membrane vesicles (MV) are, in effect, miniature representations of their parent bacterium (Beveridge, 1999). Therefore, it is little surprise that MVs from pathogens such as *P. aeruginosa* and *H. pylori* have previously been shown to elicit IL-8 release from host lung (Bauman and Kuehn, 2006; Ellis *et al.*, 2010) and gastric (Ismail *et al.*, 2003; Kaparakis *et al.*, 2010) epithelial cell lines, respectively. However, despite the high load of Gram-negative bacteria in the intestine, MVs derived from commensal bacteria are yet to be reported. Additionally, the interaction of MVs with intestinal epithelial cells have received little attention, with only one study, undertaken by Kesty *et al.* (2004), utilising MVs from ETEC, demonstrating toxin delivery to host cells, via MVs.

In this study, concentrates (up to 25x) of MVs isolated from the enteric commensal bacterium, *E. coli* C25, were used to challenge HT29-19A and Caco-2 cells. The rationale for utilising MVs at concentrations markedly higher than the culture concentrations was the fact that, *in vivo*, the overall number of Gram-negative bacteria present in the intestine is, at least, two orders of magnitude higher than that found in the bacterial mono-cultures used in this study. Therefore, MV concentrations *in vivo* are hypothesised to be much higher than those reached in *in vitro* culture systems. Nevertheless, C25-derived MVs were found to stimulate a significant increase in IL-8 expression, at both the transcriptional and translational levels, in the two cell lines, but appeared to lack anti-inflammatory activity, as they did not stimulate any detectable translational expression of IL-10.

The mechanism of interaction of MVs and host cells is currently unknown; however there are a number of possible receptors which could mediate their immunostimulatory effects. MVs are not microbe-associated molecular patterns (MAMPs) *per se*, but rather

represent a collection of MAMPs; therefore any number of cell surface PRRs, such as TLRs, could confer host cell responsiveness. The stimulation of intracellular receptors also presents a possibility, with a study by Parker *et al.* (2010) demonstrating that *H. pylori* MVs were internalised by gastric epithelial cells via clathrin-mediated endocytosis. Indeed, a recent study by Kaparakis *et al.* (2010) demonstrated that MVs isolated from *H. pylori* stimulate significant release of IL-8 from the AGS gastric epithelial cell line, via the delivery of iE-DAP, a constituent of peptidoglycan, to the intracellular NOD1 receptor.

In the present study, TLR-1, TLR-2, TLR-5 and NOD1 all showed little deviation in their mRNA expression in response to the presence of C25 MVs compared to control levels. However, C25 MVs did stimulate a complete down-regulation of TLR-9 mRNA in HT29-19A cells, a result similar to that observed earlier in the investigation, in response to pathogenic CpG DNA and to C25 sonicate (Sections 3.4 & 4.3). This effect was potentially caused by extracellular CpG-DNA, as MVs isolated from *P. aeruginosa* have previously exhibited CpG-DNA on their surface (Kadurugamuwa and Beveridge, 1995, Renelli *et al.*, 2004). Surface-associated CpG-DNA possibly contributed to the IL-8 release observed in response to C25 MVs; however, it is unlikely that it was solely responsible for the level of IL-8 stimulation provoked. Another result of interest was the down regulation of TLR4 and its co-receptor, CD14, in Caco-2 cells, in response to MVs. Desensitisation to further challenge with the TLR-4 agonist, LPS, was curious as Caco-2 cells had already been shown to lack the capacity to respond to challenge by the, LPS (Section 3.4).

Although very little modification of the mRNA expression of the receptors tested here was observed, it is possible that MVs bind the receptors, but do not affect their expression at the transcriptional level; therefore, the translational expression should also be explored via Western blot analysis. Alternatively, to investigate whether or not TLRs and/or NOD receptors mediate the IL-8 secretion in response to incubation with C25 MVs, TLR and NOD

knockout cells (utilising RNA interference (RNAi)) could be utilised to screen which receptor(s) confer responsiveness to the MVs (as would be evident by a decrease in IL-8 release). However, it is possible that the process of MV internalisation is responsible for the elicitation of IL-8, in much the same way that bacterial internalisation stimulates IL-8 release via an unknown mechanism (Eckmann *et al.*, 1993b). A protocol similar to that utilised by Bauman and Kuehn (2009) could be followed to investigate the extent of MV internalisation. They challenged lung epithelial cells with fluorescently labelled *P. aeruginosa* MVs and compared the fluorescence of challenged cells with that of control cells, where higher fluorescence was indicative of larger numbers of internalised MVs (Bauman and Kuehn, 2009).

This is the first study to consider the immunomodulatory activity of non-pathogenic MVs; therefore, their significance in the *in vivo* intestinal milieu is still very much unknown. At its maximum, IL-8 release in response to C25 MVs was relatively low compared to the level of stimulation by factors released by *C. difficile* (Figure 6.1), therefore, it can be speculated that the inflammatory response to MVs is part of the chronic low level inflammation characteristic of intestinal homeostasis. Nevertheless, to gain a more accurate representation of the extent of IL-8 stimulation by C25 MVs, they should be compared to the response to MVs isolated from Gram-negative enteropathogens, such as enterotoxigenic *E. coli* (ETEC) (Kesty *et al.*, 2004) or toxigenic *Bacteroides fragilis* (Patrick *et al.*, 1996).

6.5.2 Modulation of bacterial internalisation

Despite the fact that the intestinal epithelial cells were somewhat stressed (with moderate increases in IL-8 release observed), and the fact that increased bacterial translocation was observed in response to C25 cfs and sonicates (in Caco-2 cells), pre-treatment of cell layers with C25 MVs was shown to cause a significant decrease in transcellular bacterial

translocation. The mechanism for this is likely to be competitive inhibition, i.e. the MVs are physically blocking the receptors which mediate the internalisation of C25. However, further experimentation is required to confirm this. Additionally, it would be interesting to explore whether the inhibitory effects of pre-treatment with C25 MVs are evident with a pathogenic bacterium which possesses a greater propensity to cellular internalisation.

6.6 Commensal-derived LPS and intestinal epithelial cells

TLR-4 was previously found to be the receptor which mediates host cell responses to LPS (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999; Hirschfield *et al.*, 2000), therefore, its modulation in the presence of cell-free supernatant, sonicated bacteria and MVs all suggest that LPS plays an important role in immunomodulatory effects of *E. coli* C25.

Previous studies have revealed the sensitivity of intestinal epithelial cell lines to LPS isolated from pathogenic bacteria, with the stimulation of IL-8 in both HT29 cells (Schuerer-Maly *et al.*, 1994; Smirnova *et al.*, 2003) and Caco-2 cells (Huang *et al.*, 2003). However, this is the first study to utilise LPS isolated from a non-pathogenic, Gram-negative bacterium. Consequently, these data are the first to show the immunostimulatory ability of commensal-derived LPS with intestinal epithelial cells, with *E. coli* C25 LPS eliciting a significant increase of IL-8 expression, and release, in HT29-19A cells. Conversely, this effect was not observed in the Caco-2 cell line. The hyporesponsiveness to LPS, observed in Caco-2 cells, is concordant with results from a previous study, also undertaken in Caco-2 cells, by Abreu *et al.* (2001), and is likely to be attributable to the relatively low level of TLR-4 mRNA expression (Abreu *et al.*, 2001; Naik *et al.*, 2001).

Interestingly, despite stimulating a greater release in IL-8, in comparison to pathogenic LPS (Section 3.4), C25 LPS does not appear to induce up-regulation of TLR-4 mRNA as pathogenic LPS did (Section 3.4). With the shape of LPS lipid A regions previously being shown to be related to endotoxic activity (Schroemm *et al.*, 2000; Netea *et al.*, 2002), it is possible that commensal and pathogenic bacteria express LPSs with different lipid A conformations, leading to differences in immunomodulatory activity. To investigate the structure of the lipid A moieties of LPS molecules, previous studies have utilised analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy (Strain *et al.*, 1983; Ribeiro *et al.*, 1999), matrix-assisted laser desorption/ionization time-of-flight mass

spectrometry (MALDI-TOF MS) (Lindner, 2000), small angle X-ray diffraction (SAXD) (Schromm *et al.*, 2000). Therefore, these techniques could be used in further experimentation, aiming to discern the structure of C25 LPS and could compare it to LPSs from known pathogenic strains of *E. coli*. Conversely, is unreasonable to exclude the potential for such contamination to be a contributing factor in the immunostimulatory activity of C25 LPS. Previous studies have reported stimulation of human macrophages and embryonic kidney cells by LPS, via TLR-2 (Kirschning *et al.*, 1998; Yang *et al.*, 1998), which was subsequently found to be attributable to contamination of LPS samples with endotoxic proteins (Hirschfield *et al.*, 2000). Due to time restrictions, this was not explored in the present study, however, any such contaminating proteins could be removed by a multi-step precipitation process (Hirschfield *et al.*, 2000).

A study undertaken by Berg *et al.* (1995) demonstrated that, *in vivo*, LPS isolated from pathogenic *E. coli* was able to induce the secretion of the anti-inflammatory cytokine, IL-10, suggesting a mechanism for the phenomenon of LPS tolerance. Nevertheless, this effect was not observed in the *in vitro* system of this investigation, as no detectable levels of IL-10 were released by either cell line in response to C25 LPS.

Host cell responsiveness to LPS requires the presence of serum (Patrick *et al.*, 1992), specifically LPS binding protein (LBP), which opsonises LPS molecules chaperones LPS to the CD14 co-factor (Hailman *et al.*, 1994). CD14 subsequently presents the bound LPS molecules to TLR-4, which consequently mediates the cellular immune response (Triantafilou and Triantafilou, 2002). In serum-free conditions, the IL-8 release observed in C25 LPS-challenged HT29-19A cells was comparable to levels in control cells (also treated with serum-free media), thus confirming that the proinflammatory activity of C25 LPS was serum-dependent. Additionally, in the absence of serum, a significant reduction in IL-8 release (compared to challenge in the presence of serum) was observed in HT29-19A cells

challenged with C25 cfs, thus suggesting that LPS, or indeed another serum-dependent antigen, was a major contributing factor in the proinflammatory activity of C25 cfs. *In vivo*, this effect could have major implications in the pathogenesis of IBD. Increased lumenal concentrations of serum proteins, such as LBP, resulting from the ‘leaky’ epithelium, often associated with IBD (Schmitz *et al.*, 1999; Soderholm *et al.*, 2002), could potentially result in augmented immune responses to commensal-derived LPS molecules, thus providing a mechanism to drive the inflammatory disease.

Despite its inflammatory nature, pre-treatment with C25 LPS did not cause any significant differences in bacterial translocation in either of the two cell lines. These data indicate that the ability to stimulate an increase in bacterial translocation is more complex than inflammatory activity, as previously alluded to (see Section 6.4.1).

6.7 Probiotic potential of extracellular products from two previously uncharacterised lactobacilli strains

Lactobacilli naturally reside in the adult human intestine (Lebeer *et al.*, 2008) and are found in a wide range of fermented food products, such as milk drinks, fruit juices and cheeses, either as members of the natural bacterial content, or as deliberately administered probiotic cultures (Turpin *et al.*, 2010). One of the major health benefits of probiotic lactobacilli is their anti-inflammatory activity (Nandakumar *et al.*, 2008; Moorthy *et al.*, 2010; Seifert *et al.*, 2010; Stober *et al.*, 2010). This beneficial immunomodulatory activity could be ascribed to probiotics inhibiting receptor sites within the intestine, thus blocking further bacterial binding; however, it seems unlikely that this could account for the alleviation of excessive inflammation in pre-existing conditions, such as IBD (Madsen *et al.*, 1999; Mimura *et al.*, 2004; McCarthy *et al.*, 2003). Therefore, it is hypothesised that probiotics possess more specific anti-inflammatory properties, nevertheless, most studies in this field have only utilised the live organisms themselves, and have not considered the extracellular products secreted by these bacteria. The initial experiments in this study were designed to investigate if the soluble factors isolated from the previously unstudied lactobacilli strains *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski (Rosy), had immunomodulatory activity on intestinal epithelial cell lines HT29-19A and Caco-2.

The effects of the untreated MRS broth were initially investigated, and, although a stimulation of IL-8 release was observed in Caco-2 cells, there was a distinct qualitative difference between the untreated broth and the cell-free supernatants (cfs's) from the two strains of lactobacilli, therefore, this approach was pursued. Lactobacilli cfs's were shown to elicit a small, but significant, increase in both expression and release of IL-8, from the Caco-2 cell line; however, only the 5e2 strain exhibited this activity with HT19-19A cells. Extracellular products from either strain of *Lactobacillus* were unable to stimulate the release

of IL-10, and this was also reflected at the transcriptional level. Indeed, the 5e2 cell-free supernatant actively down-regulated IL-10 mRNA expression in Caco-2 cells.

Due to its abundance in the Gram-positive cells (Schleifer and Kandler, 1972), the primary antigen likely to be released by ultrasonic disruption of lactobacilli would be peptidoglycan (Section 1.5.8); however, other antigens, such as CpG DNA (Section 1.5.9), lipoproteins (Section 1.5.10) and lipoteichoic acid (Section 1.5.11), would also be liberated. In response to lactobacilli sonicates, IL-8 release was stimulated in Caco-2 cells and was of greater magnitude than that elicited by cell-free supernatants, thus further supporting the hypothesis that ultrasonic disruption increases antigenic release from bacteria. However, HT29-19A cells appeared unresponsive to sonicates of either strain *Lactobacillus*, indicating either that the IL-8-stimulating antigen in the 5e2 cell-free supernatant was denatured or an additional factor was released by sonication and was attenuating the release of IL-8.

The up-regulation of TLR-1 and TLR-2 mRNAs in HT29-19A cells, in response to challenge with 5e2 cfs, suggested that these receptors were directly stimulated by agonists, such as peptidoglycan and lipoteins. Furthermore, the complete down-regulation of TLR-9 mRNA also observed was characteristic of CpG-DNA binding (Section 3.4); therefore, HT29-19A cell stimulation was also potentially mediated via TLR-9. Conversely, and consistent with the lack of IL-8 release, HT29-19A cells exhibited a much diminished modulation of TLR mRNA in response to Rosy cfs, compared to that observed in response to 5e2 cfs. Nevertheless, in concordance with the effects of 5e2 cfs, TLR-2 mRNA was significantly up-regulated and TLR-9 was completely down-regulated. These results implied that TLR-2 and TLR-9 agonists were present in Rosy cfs, thus provoking the up-regulation of their respective receptors, but were not subsequently stimulating IL-8 release. Should these results be paralleled in an *in vivo* system, then they could imply inflammation-independent priming (via the modulation of TLR expression) of the epithelial layer by certain food-borne

bacteria.

In response to challenge with 5e2 or Rosy cfs, Caco-2 cells exhibited very little deviation from control level of TLR mRNA. The exception to this was a significant down-regulation of TL-9 mRNA in response to 5e2 cfs, a result consistent with stimulation by CpG-DNA, and concordant with the effects of 5e2 cfs in HT29-19A cells. It is likely that peptidoglycan, the major constituent of the Gram-positive cell wall, contributed to the stimulation of Caco-2 cells, but the cell line's high constitutive expression of TLR-2 meant that they were likely to be less susceptible to up-regulation. However, in response to challenge with 5e2 sonicate, Caco-2 cells did exhibit a small, but statistically significant, up-regulation of TLR-2 mRNA; nevertheless, the hypothesised increased concentrations of soluble peptidoglycan could account for this. Additionally, a similar pattern was also observed in response to Rosy sonicate.

The use of probiotic bacteria, particularly strains of lactobacilli, has previously shown success in the treatment of patients suffering antibiotic- and *C. difficile*-associated diarrhoea (Hickson *et al.*, 2007; Doron *et al.*, 2008; Gao *et al.*, 2010). Additionally, a study undertaken by Banerjee *et al.* (2009) demonstrated that the secretory products of *Lactobacillus delbrueckii* sp. *bulgaricus* were able to attenuate the cytotoxic activity of *C. difficile* toxins when Caco-2 cells were challenged with various cocktails of cell-free supernatants from cultures of both bacteria. The data presented by Banerjee *et al.* (2009) showed that the extracellular products from non-pathogenic bacteria can directly reduce the effects of pathogens via a mechanism independent from out-competition by the live bacteria. The potential of the strains of lactobacilli, investigated in the present study, acting in this manner, was explored utilising sequential treatment of epithelial cells with lactobacilli cell-free supernatant, followed by challenge with *C. difficile* cell-free supernatant. However, there was

no indication that either strain of lactobacilli had any protective effects against the inflammatory activity of *C. difficile* cell-free supernatant.

Taken all together, these results suggested that extracellular products from *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski did not demonstrate probiotic potential. However, to some extent, this study was confounded by the stimulatory effects of the MRS broth on the Caco-2 cell line. Nevertheless, this study has concentrated on just two of the numerous inflammatory cytokines expressed in intestinal epithelial cells, and therefore further investigation is required to confirm this.

6.8 EPSs and intestinal epithelial cells

6.8.1 Immunomodulatory activity

Having previously observed the confounding effects of the MRS broth on the Caco-2 cell line, it was decided to investigate the immunomodulatory effects of purified EPSs produced by *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski. The immunomodulatory effects of EPSs on intestinal epithelial cells have little been studied thus far, with only Hidalgo-Cantabrana and colleagues, recently considering the concept and preliminarily demonstrating differential effects of EPS-producing bifidobacteria on the release of inflammatory cytokines, such as IL-6 and IL-8, in the Caco-2 cell line (Hidalgo-Cantabrana *et al.*, 2012).

The purified EPSs tested in this investigation were shown to provoke a small, yet statistically significant, increase in IL-8 expression and release in HT29-19A cells. Additionally, 5e2 EPS was able to significantly up-regulate IL-8 mRNA in Caco-2 cells, although this was not paralleled at the translational level. In contrast to this, neither EPS was able to stimulate IL-10 expression, at either the transcriptional or translational level, in either of the two cell lines. These initial results indicate that EPSs are able to stimulate a low-level proinflammatory response in cultured intestinal epithelial cells. If this effect was also to occur *in vivo*, it would suggest a potential mechanism of action in intestinal homeostasis. For example, the low level inflammation caused by these EPSs could compete with more potent inflammatory mediators, thus protecting against excessive inflammation. In concordance with this hypothesis, a study by Sengül *et al.* (2006) demonstrated that EPS-producing bacteria were able to significantly attenuate the inflammation of an experimental colitis model, induced via intracolonic administration of acetic acid, in rats. Alternatively, the low level of IL-8 secretion reported here could contribute to the theorised epithelial ‘priming’ effect of the commensal microflora, *in vivo*, which allows elicitation of a more controlled and rapid host response to pathogenic attack (Macpherson and Harris, 2004; Artis, 2008).

Currently, information on the mechanism of interaction of EPSs with host cells is limited, however, a recent study on a novel EPS (TA-1) isolated from the thermophilic marine bacterium, *Thermus aquaticus*, was shown to stimulate the release of proinflammatory cytokines, TNF- α and IL-6, from murine macrophages via a TLR-2-mediated pathway (Lin *et al.*, 2011). This is consistent with the fact that TLR-2 is well characterised in its interactions with a diverse range of microbial components (Takeda *et al.*, 2003, Akira *et al.*, 2006). When challenged with the EPSs considered in this study, HT29-19A cells exhibited a significant up-regulation of TLR-2 mRNA expression. This study, and earlier ones (Politorak *et al.*, 1998; Visintin *et al.*, 2001; Hornung *et al.*, 2002), have previously shown that, upon binding, TLR agonists cause the up-regulation of their cognate receptors, therefore, it can be speculated that the up-regulation of TLR-2 mRNA in HT29-19A cells is due to direct association with the EPSs. It has also been shown that the up-regulation of TLR-2 also appeared to sensitise HT29-19A cells to subsequent challenge with the known TLR-2 agonist, peptidoglycan (PGN), with a small, but statistically significant, increase in the IL-8 secretion. Contrary to this, no significant change in TLR-2 mRNA was detected in the Caco-2 cell line. However, it was previously observed that Caco-2 cells possess a much increased constitutive expression of TLR-2 (Section 3.2) in comparison to HT29-19A cells; therefore, the up-regulatory effects could be masked. Nevertheless, despite having hypothesised that the EPSs only directly associate with TLR-2, the modulation of the expression of other TLRs was also considered.

Previously, live lactobacilli have been shown to up-regulate expression of non-cognate TLRs in intestinal epithelial cells, thus sensitising them to subsequent challenge with bacterial antigens (Viszoso Pinto *et al.*, 2009; Seifert *et al.*, 2010). The same phenomenon was observed in this investigation, with Rosy EPS sensitising the HT29-19A cell line to subsequent challenge with the TLR-5 agonist, bacterial flagellin, causing a significant

increase in IL-8 release. This sensitisation could potentially be of clinical importance because flagellin plays an important role in gut immunity, and is known to be involved in both homeostatic regulation of immune responses to the commensal microflora (Vijay-Kumar *et al.*, 2008) and the pathogenesis of inflammatory bowel disease (Lodes *et al.*, 2004; Targan *et al.*, 2005). Indeed, Lodes *et al.* (2004) showed flagellin to be a dominant antigen in patients with Crohn's disease. The mechanism for the sensitisation of epithelial cells to bacterial products is currently unknown; however, it is thought that stimulation of the common intracellular pathways involved in transduction of TLR-stimulated signals may be responsible (Viszoso Pinto *et al.*, 2009).

The potential for EPSs priming the epithelial layer to pathogen attack by TLR-independent stimulation was also tested using the pre-treatment of epithelial cell lines with EPSs, prior to challenge with *C. difficile* cell-free supernatant. The results of this showed that neither EPS had any significant effects on IL-8 release in response to *C. difficile* cell-free supernatant. Therefore, it can be hypothesised that the epithelial priming activities of EPSs are mediated through the modulation of TLR expression.

Preliminary data published by Hidalgo-Cantabrana *et al.* (2012) demonstrated the stimulation of inflammatory cytokines, in Caco-2 cells, in response to co-culture with EPS-producing bifidobacteria. However, the present study showed, for the first time, that purified EPSs were able to directly interact with intestinal epithelial cells, stimulating IL-8 expression at both the transcriptional and translational level. Additionally, it showed that EPSs modulated TLR mRNA expression in intestinal epithelial cell lines, resulting in 'priming' of HT29-19A cells and a potentiated release of IL-8 in response to subsequent challenge with bacterial antigens, peptidoglycan and flagellin.

6.8.2 Barrier function of Caco-2 monolayers

Live lactobacilli have previously been shown to increase intestinal epithelial integrity, causing an increase in trans-epithelial electrical resistance (TEER) (Nissen *et al.*, 2009). As indicated by domes, the results of this study showed that 5e2 EPS had no significant effect on the integrity of Caco-2 monolayers. Conversely, and in contrast to the results from the study by Nissen *et al.* (2009), Rosy. EPS appeared to cause a temporary loosening of the tight junction in Caco-2 cells, as suggested by the short-term loss of domes. The effects were apparent immediately after incubation with the EPS; however, the decrease in barrier integrity was reversed following removal of the EPS. The mechanism for this could potentially be mediated via a TLR-2 pathway, as this receptor has been shown to regulate epithelial barrier function (Cario *et al.*, 2007); however, much more investigation is required.

6.8.3 Bacterial adherence and internalisation

Adherence is a key step in bacterial colonisation of host mucosal surfaces (Beachey, 1981). A study undertaken by Lebeer *et al.* (2007) demonstrated a key role for EPSs in bacterial attachment to abiotic surfaces, however, the role of EPSs in attachment of bacteria to biological moieties is unclear. EPSs have been shown to promote the adherence of pathogenic bacteria to intestinal mucus (Ruas-Madiedo *et al.*, 2006a; Ruas-Madiedo *et al.*, 2006b); however, these same EPSs were also shown to inhibit the attachment of probiotic bacteria to the mucus (Ruas-Madiedo *et al.*, 2006a; Ruas-Madiedo *et al.*, 2006b). Here, the modulatory potential of EPSs on the direct attachment, and subsequent internalisation of *E. coli* C25 on intestinal epithelial cells was explored.

The data obtained in this investigation showed that 5e2 EPS was unable to provoke any significant changes in either adherence or internalisation of *E. coli* C25, in either HT29-19A or Caco-2 cells. However, Rosy EPS exhibited modulatory activity with Caco-2 cells,

causing a significant increase in adherence of *E. coli* C25, but having no subsequent effects on internalisation. This effect could suggest that Rosy EPS was able to form a protective, ‘sticky’ coating, akin to intestinal mucus, on Caco-2 cells. In contrast to this and possibly due to the differences in cell line origin, HT29-19A cells showed a significant increase in bacterial internalisation without a significant increase in adherence of C25, following treatment with Rosy EPS. However, should this effect be seen *in vivo*, it could represent a mechanism via which the transcellular translocation of commensal bacteria is actively promoted, resulting in the development of host immunocompetency (Lichtman *et al.*, 2001) and microfloral tolerance (Mowat, 2003). Finally, it is worth noting that these effects were not attributable to increased proliferation of C25 in the presence of EPSs, as no significant differences in bacterial populations were observed, compared to EPS-free controls.

6.8.4 Future work

A major limitation of the work in this study was the availability of the EPSs, due to both their limited release by the bacteria and the laborious nature of the purification process. Had they been more easily obtainable, the investigation could have been taken much further. A logical starting point for future work would be the translational confirmation of the changes in TLR expression, seen in this investigation, in response to EPSs. This could be undertaken utilising Western blot analysis or flow cytometry. Also, the working concentration of EPSs used in this study was a ‘mid-range’ concentration taken from the existing literature and so, given larger volumes of EPSs, a range of concentrations could be tested to explore the extent of their activity at higher and lower doses. Moreover, experimentation similar to that undertaken by Lin *et al.* (2011) could be utilised to in an attempt to determine if TLR-2 is the receptor responsible for intestinal epithelial cell responsiveness to EPSs. A TLR-deficient cell line, such as the human embryonic kidney (HEK)293T cell line used by Lin and colleagues, and

its subsequent transfection with TLR-2 could be utilised to investigate whether or not TLR-2 does in fact confer responsiveness to the EPSs (as would be evident by an increase in IL-8 secretion, compared to control cells). Additionally, blocking of intestinal epithelial cells with anti-TLR-2 antibodies prior to stimulation with EPSs could also be used to substantiate, via a decrease in IL-8 secretion, that TLR-2 confers responsiveness to EPSs.

6.9 Immunomodulatory effects of bacterial extracellular products

Taken all together, the results of this investigation indicate that, in the two cell lines utilised, the mRNA expression of the TLR receptors is, in fact, representative of their functionality. Owing to its increased expression of TLR-4 mRNA, the HT29-19A cell line is more responsive to the products isolated from the Gram-negative bacterium, *E. coli* C25. Conversely, the Caco-2 cell line, which expressed higher levels of TLR-2 mRNA, was much more sensitive to the extracellular products isolated from the Gram-positive lactobacilli strains. Additionally, the magnitude of IL-8 release from both cell lines, in response to the range of non-pathogenic extracellular products tested, was relatively low in comparison with the response observed in response to extracellular products from *C. difficile* (Figure 6.1). This moderate level of inflammation is potentially accountable for the theorised epithelial ‘priming’ effect of the commensal microflora (Macpherson and Harris, 2004; Artis, 2008).

However, the physiological relevance of the results gained in this investigation is somewhat limited by the incomplete polarisation of the intestinal epithelial cell lines utilised. *In vivo*, intestinal epithelial cells are fully polarised, subsequently differentiating into the apical and basolateral surfaces. To achieve polarisation in *in vitro* cell lines, they must be cultured on Transwell™ permeable inserts (Corning, UK); however, due to a lack of resources, they were unavailable for the current study. The use of fully polarised monolayers is more physiologically accurate to the enteric conditions, with intestinal epithelial cell lines differentially responding to apical and basolateral stimulation (Lammers *et al.*, 1994; Lee *et al.*, 2006), as would be seen *in vivo* (Gewirtz *et al.*, 2001).

Another key limitation of this investigation is the fact that TLR expression was only explored at the mRNA level and, given that the relationship between transcriptional and translational expression is hypothesised to be relatively weak (Schwanhäusser *et al.*, 2011), changes in TLR mRNA, in response to bacterial products, may not be wholly representative

of changes in protein expression. Therefore, an obvious starting point for future work is to validate the mRNA expression changes observed in this study by investigating the translational expression of TLRs in response to the range of bacterial products investigated. This could be achieved by utilising techniques such as Western blot analysis and/or flow cytometry.

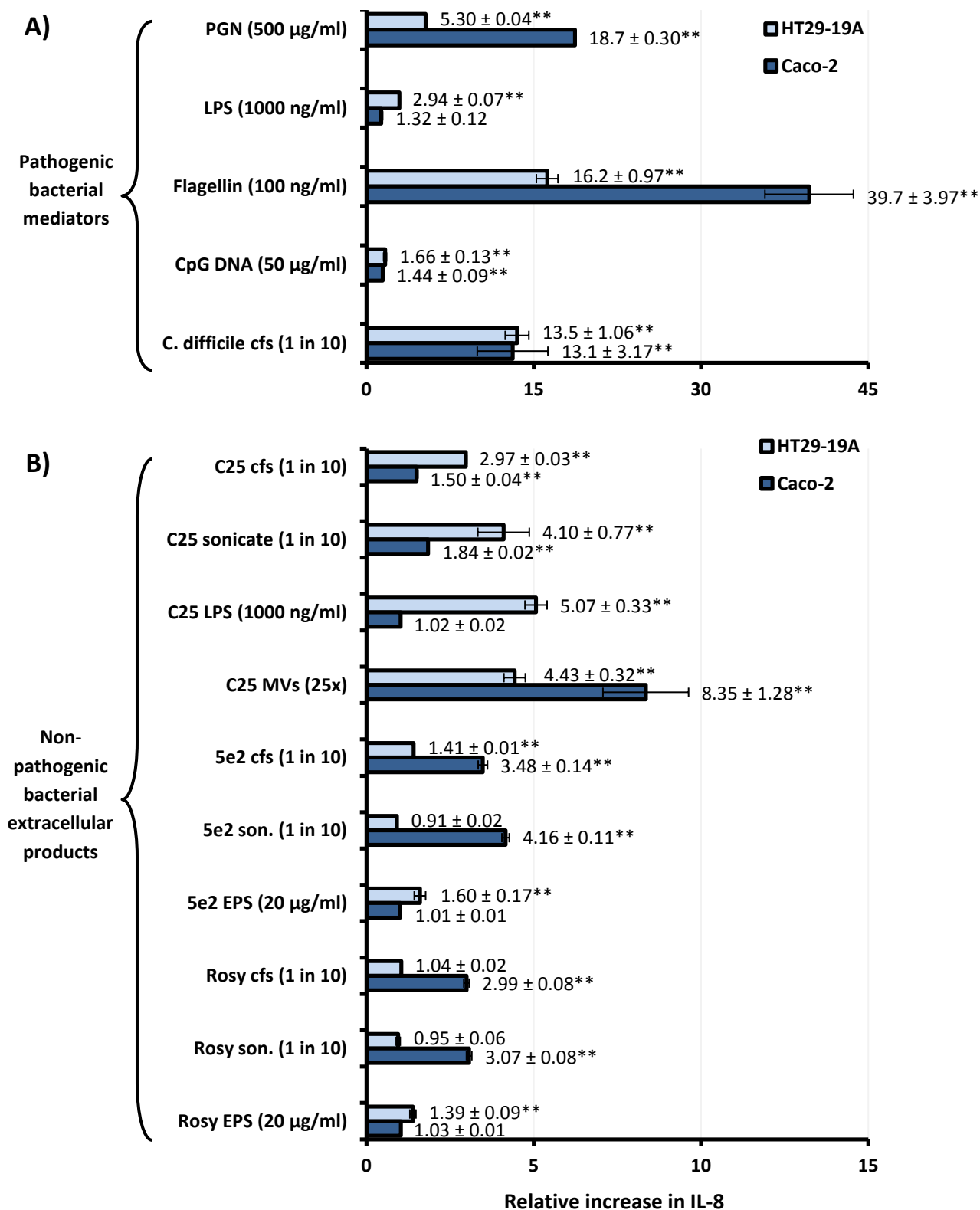


Figure 6.1 – Comparing the magnitude of IL-8 release, in HT29-19A and Caco-2 cells, in response to bacterial extracellular products. Cells were incubated with various pathogenic (A) or non-pathogenic (B) challenge samples for 24 h and IL-8 was measured. Results are fold change in IL-8 release, relative to the constitutive level.

6.10 General summary

The symbiosis that exists between the intestinal microflora and the human host is clearly a highly complex relationship that upholds a very fine balance between health and disease. Studies utilising germfree animals have demonstrated that the microflora is essential in the maturation and functionality of the intestine, with its absence leading to significantly underdeveloped intestinal walls and mucosal immune systems. Paradoxically, the commensal enteric microflora is also a vast source of antigenic materials which relentlessly challenge the intestinal innate immunity, resulting in a state of constant immunological stimulation. Furthermore, the microflora, and its associated antigens, has also been heavily implicated in the aetiology of chronic inflammatory diseases, such as Crohn's disease and ulcerative colitis. However, in healthy individuals, the innate immune system is able to maintain a multifaceted homeostatic balance between remaining hyporesponsive to the commensal microflora, yet, also retaining the capacity to react to pathogenic attack. The key interface in the regulatory defence system is the intestinal epithelial layer, which forms both a physical barrier and an innate immune sensor. The expression of pattern recognition receptors in epithelial cells allows the detection of a wide range of conserved microbial products released by both pathogenic and non-pathogenic bacteria. Nevertheless, previous studies have generally only characterised the immunological activities of extracellular products from pathogenic or known probiotic bacteria, thus neglecting the vast populations of bacteria between the two extremes. The present study is one of the first investigations characterising the interactions of the intestinal epithelial layer with products from bacteria not previously recognised to be pathogenic or probiotic. Although work undertaken in this study was non-mechanistic in nature, it has provided new information on the role of the enteric-associated bacteria in intestinal homeostasis, highlighting the immunomodulatory potential of their extracellular products. Additionally it has provided novel evidence that non-pathogenic extracellular

products could play a role in the development of host tolerance to the commensal microflora.

The HT29-19A and Caco-2 cell lines exhibited very different expression levels of IL-8; with HT29-19A cells possessing a much larger capacity, than Caco-2 cells, for constitutive release of the proinflammatory chemokine. Conversely, despite a lack of detectable protein production, Caco-2 cells exhibited the potential for the secretion of the potent anti-inflammatory cytokine, IL-10, with mRNA expression evident, whereas HT29-19A cells did not exhibit detectable signals in either transcription or translation. Additionally, the TLR mRNA expression profiles of the cell lines were very distinct from one another, which, consequently, had profound effects on their sensitivity to known bacterial agonists. The two cell lines exhibited similar levels of mRNA expression for TLR-5 and TLR-9, and subsequently demonstrated comparable sensitivities to both flagellin and CpG-DNA. However, HT29-19A cells exhibited much higher mRNA expression of TLR-4, than Caco-2 cells, and consequently demonstrated a much higher sensitivity to the TLR-4 agonist, LPS. Additionally, Caco-2 cells were found to possess significantly increased levels of TLR-2 mRNA, in comparison to HT29-19A cells, thus exhibiting a much increased sensitivity to the TLR-2 agonist, PGN. In contrast to this, both cell lines exhibited an increase, of comparable magnitude, in IL-8 release, in response to the products of the enteropathogen, *C. difficile*, which are thought to act in a TLR-independent manner. Finally, Caco-2 cells were shown to form physiologically active tight junctions, with the formation and maintenance of domes.

Although this investigation was not the first to demonstrate the proinflammatory effects of *E. coli* C25 cfs and sonicate, the extended cell challenge utilised here was much more representative of an *in vivo* situation. Also, unlike previous studies, the immunomodulatory effects of C25 were explored at the transcriptional level in both HT29-19A and Caco-2 cells. Additionally, IL-10 expression was investigated, with significant up-regulation of IL-10 mRNA observed in both cell lines, suggesting a role in intestinal

homeostasis; however, this effect was not reflected at the translational level. Also, evidence is presented that C25 LPS is a major contributing factor to the proinflammatory nature of C25 extracellular products, stimulating IL-8 release from HT29-19A cells. Moreover, MVs isolated from C25 were shown to possess immunomodulatory activity with both HT29-19A and Caco-2 cells, stimulating IL-8 and modulating TLR mRNA expression. Consequently, this thesis was the first study to consider the immunomodulatory effects of commensal-derived MVs on intestinal epithelial cells. Finally, the extracellular products were shown to regulate bacterial internalisation, in both HT29-19A and Caco-2 cells, with cfs and sonicate significantly potentiating the internalisation of *E. coli* C25, but MVs were found to somewhat inhibit the process.

Additionally, this thesis was the first to investigate the immunomodulatory effects of extracellular products, specifically ultrapurified EPSs, from two previously unstudied, EPS-producing bacteria *L. acidophilus* 5e2 and *L. helveticus* sp. Rosyjski. Crude cell-free supernatants and bacterial sonicates from two the lactobacilli strains were found to be biologically active, stimulating a relatively moderate, yet statistically significant, IL-8 release, in the Caco-2 cell line. However, the stimulatory effects were less evident in HT29-19A cells. Nevertheless, EPSs isolated and ultrapurified from the two strains exhibited novel immunomodulatory effects on HT29-19A cells, with IL-8 release and TLR modification observed. EPSs demonstrated fewer effects on Caco-2 cells, yet, TLR-modification was still evident. Pre-treatment with EPSs were also found to 'prime' HT29-19A cells to the bacterial antigens, peptidoglycan and flagellin, significantly potentiating the resultant release in IL-8. Additionally, EPSs were found to modify bacterial adherence and internalisation in both cell lines.

In conclusion, data presented in this investigation has shown that the use of two phenotypically divergent, yet highly complementary, *in vitro* intestinal epithelial cell lines

HT29-19A and Caco-2 present a reasonable model for investigating the interaction of bacterial extracellular products with the intestinal epithelium. Additionally, it has been one of the first studies to demonstrate that extracellular products, isolated from non-pathogenic, enteric-associated bacteria, stimulate a relatively mild proinflammatory response from, and modulate TLR mRNA expression in, intestinal epithelial cell lines. Furthermore, for the first time, the potential for non-pathogenic bacterial products to increase bacterial internalisation in intestinal epithelial cells has been shown. If the novel effects observed in this investigation also occurred *in vivo*, then they could potentially contribute to intestinal homeostasis, the innate ‘priming’ of the epithelial layer to pathogens and their products or even the development of host tolerance to the commensal microflora.

Chapter 7

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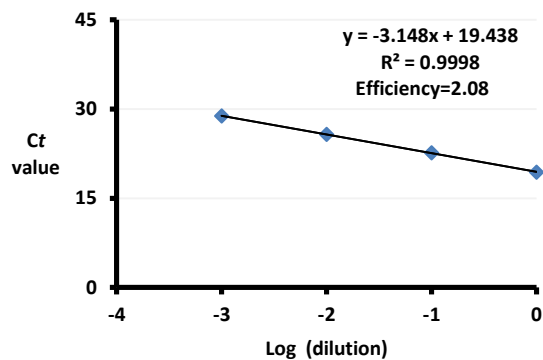
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Chapter 8

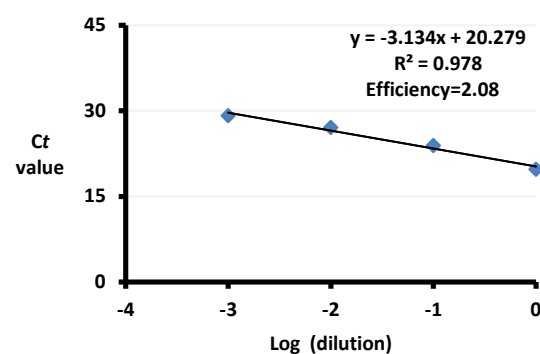
Appendices

8.1 Validating PCR primers

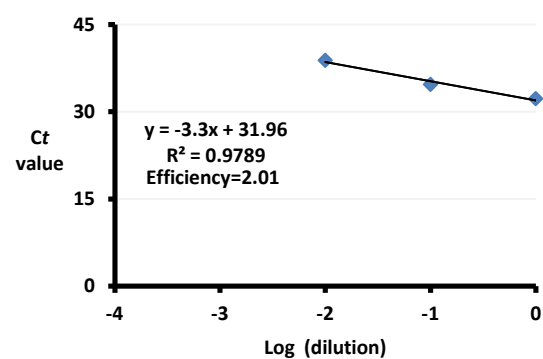
A) Actin



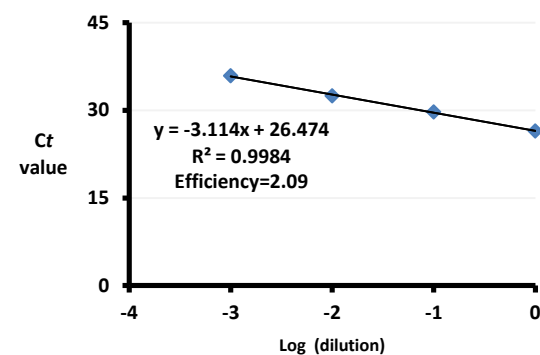
B) GAPDH



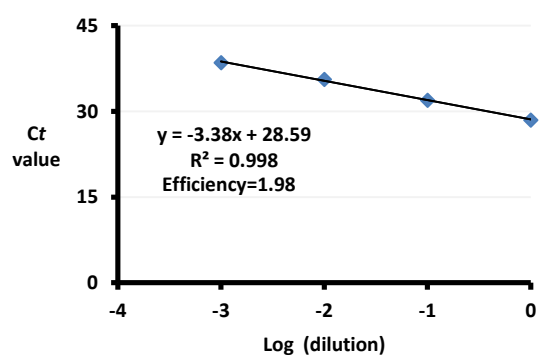
C) TLR-1



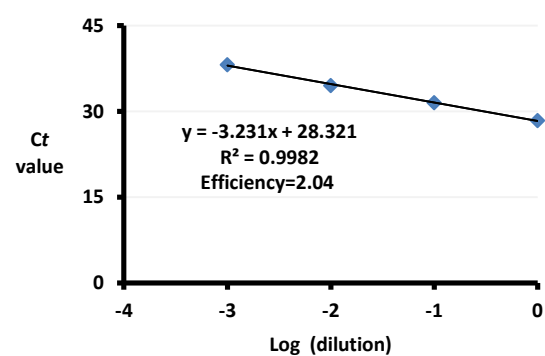
D) TLR-2



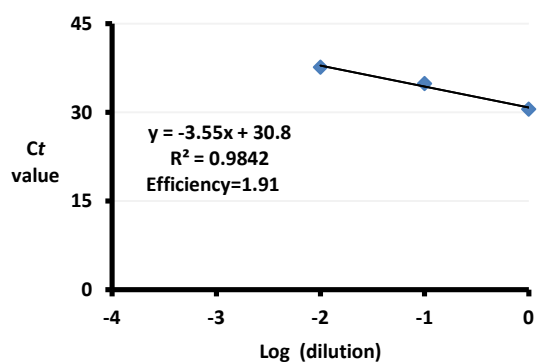
E) TLR-4



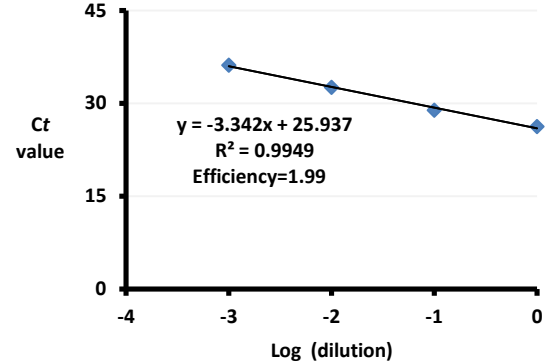
F) TLR-5



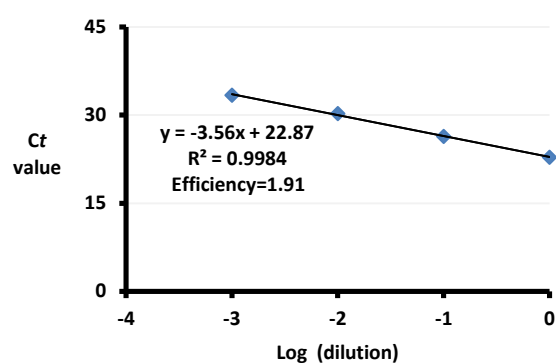
G) TLR-9



H) CD14



I) IL-8



J) NOD1

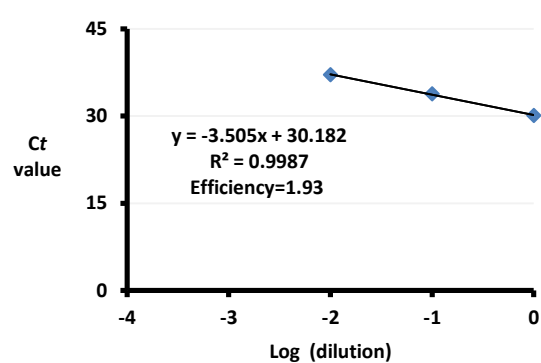


Figure 8.1 – PCR primer efficiency validation. cDNA samples were serially diluted and subjected to qRT-PCR. The resultant Ct values were plotted against the \log_{10} of the dilution.

8.2 Characterising the proliferation of HT29-19A and Caco-2 cell lines

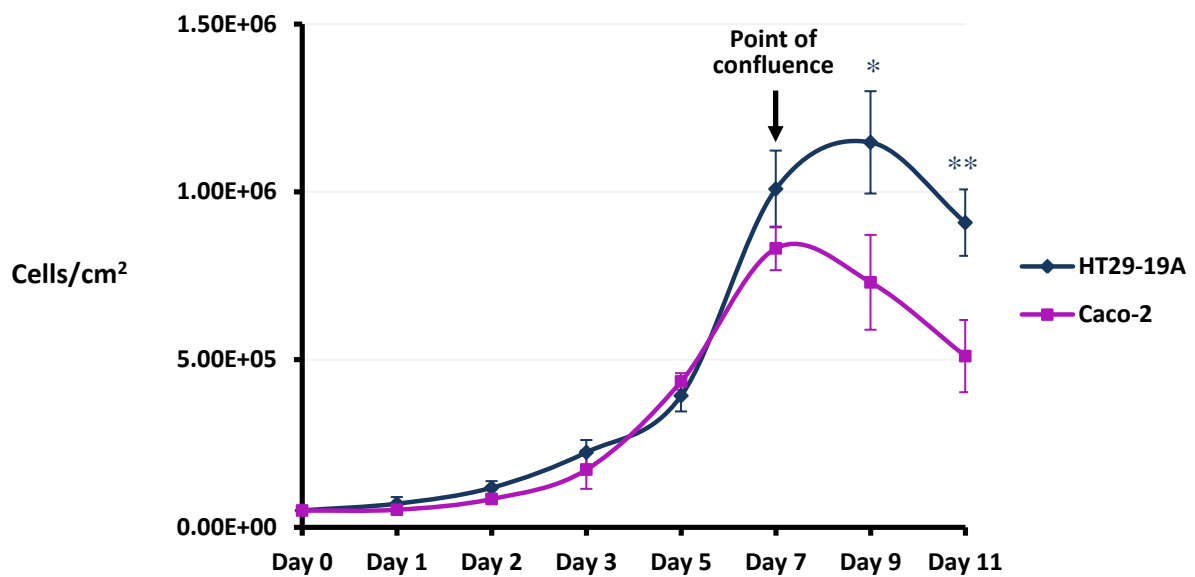


Figure 8.2 – HT29-19A cells grow to a higher cell density than Caco-2 cells. Cells were seeded at 0.5×10^5 cells/cm² and were counted at regular time points utilising trypan blue dye and a haemocytometer. Results are mean \pm SEM, n = 3.

8.3 Characterisation of *E. coli* C25

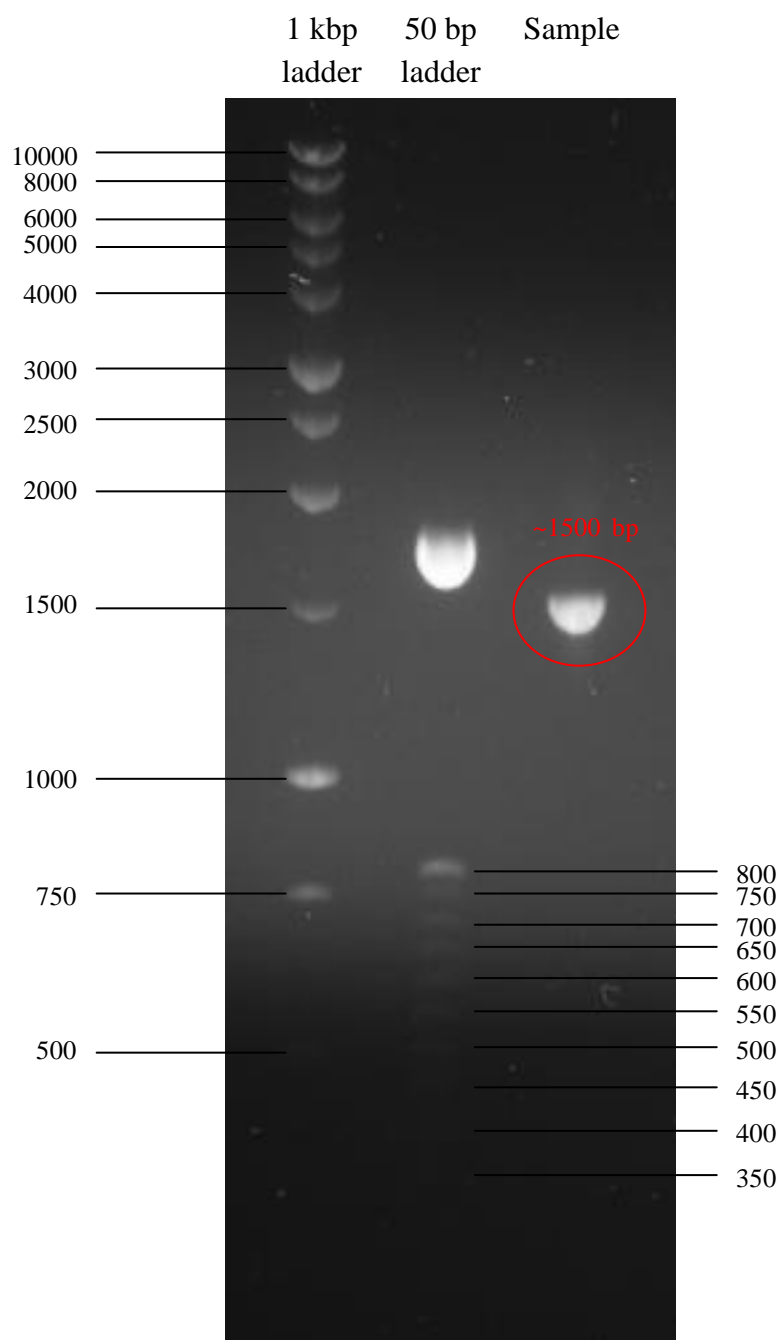


Figure 8.3 – The bacterial 16S gene is amplified by PCR. The total DNA was isolated from the bacterial culture and subjected to PCR to amplify the 16S gene. Following PCR, the product was subjected to gel electrophoresis. The resulting band is circled in red.

GAT	GGA	CGC	TGG	CGG	CAG	GCC	TAA	CAC	ATG	CAA	GTC	GAA	CGG	TAA
CAG	GAA	ACA	GCT	TGC	TTT	TTT	GCT	GAC	GAG	TGG	CGG	ACG	GGT	GAG
TAA	TGT	CTG	GGA	AAC	TGC	CTG	ATG	GAG	GGG	GAT	AAC	TAC	TGG	AAA
CGG	TAG	CTA	ATA	CCG	CAT	AAC	GTC	GCA	AGA	CCA	AAG	AGG	GGG	ACC
TTC	GGG	CCT	CTT	GCC	ATC	GGA	TGT	GCC	CAG	ATG	GGA	TTA	GCT	AGT
AGG	TGG	GGT	AAC	GGC	TCA	CCT	AGG	CGA	CGA	TCC	CTA	GCT	GGT	CTG
AGA	GGA	TGA	CCA	GCC	ACA	CTG	GAA	CTG	AGA	CAC	GGT	CCA	GAC	TCC
TAC	GGG	AGG	CAG	CAG	TGG	GGA	ATA	TTG	CAC	AAT	GGG	CGC	AAG	CCT
GAT	GCA	GCC	ATG	CCG	CGT	GTA	TGA	AGA	AGG	CCT	TCG	GGT	TGT	AAA
GTA	CTT	TCA	GCG	GGG	GGA	AGG	GAG	TAA	AGT	TAA	TAC	CTT	TGC	TCA
TTG	ACG	TTA	CCC	GCA	GAA	GAA	GCA	CCG	GCT	AAC	TCC	GTG	CCA	GCA
GCC	GCG	GTA	ATA	CGG	AGG	GTG	CAA	GCG	TTA	ATC	GGA	ATT	ACT	GGG
CGT	AAA	GCG	CAC	GCA	GGC	GGT	TTG	TTA	AGT	CAG	ATG	TGA	AAT	CCC
CGG	GCT	CAA	CCT	GGG	AAC	TGC	ATC	TGA	TAC	TGG	CAA	GCT	TGA	GTC
TCG	TAG	AGG	GGG	GTA	GAA	TTC	CAG	GTG	TAG	CGG	TGA	AAT	GCG	TAG
AGA	TCT	GGA	GGA	ATA	CCG	GTG	GCG	AAG	GCG	GCC	CCC	TGG	ACG	AAG
ACT	GAC	GCT	CAG	GTG	CGA	AAG	CGT	GGG	GAG	CAA	ACA	GGA	TTA	GAT
ACC	CTG	GTA	GTC	CAC	GCC	GTA	AAC	GAT	GTC	GAC	TTG	GAG	GTT	GTG
CCC	TTG	AGG	CGT	GGC	TTC	CGG	AGC	TAA	CGC	GTT	AAG	TCG	ACC	GCC
TGG	GGA	GTA	CGG	CCG	CAA	GGT	TAA	AAC	TCA	AAT	GAA	TTG	ACG	GGG
GCC	CGC	ACA	AGC	GGT	GGA	GCA	TGT	GGT	TTA	ATT	CGA	TGC	AAC	GCG
AAG	AAC	CTT	ACC	TGG	TCT	TGA	CAT	CCA	CGG	AAG	TTT	TCA	GAG	ATG
AGA	ATG	TGC	CTT	CGG	GAA	CCG	TGA	GAC	AGG	TGC	TGC	ATG	GCT	GTC
GTC	AGC	TCG	TGT	TGT	GAA	ATG	TTG	GGT	TAA	GTC	CCG	CAA	CGA	GCG
CAA	CCC	TTA	TCC	TTT	GTT	GCC	AGC	GGT	CCG	GCC	GGG	AAC	TCA	AAG
GAG	ACT	GCC	AGT	GAT	AAA	CTG	GAG	GAA	GGT	GGG	GAT	GAC	GTC	AAG
TCA	TCA	TGG	CCC	TTA	CGA	CCA	GGG	CTA	CAC	ACG	TGC	TAC	AAT	GGC
GCA	TAC	AAA	GAG	AAG	CGA	CCT	CGC	GAG	AGC	AAG	CGG	ACC	TCA	TAA
AGT	GCG	TCG	TAG	TCC	GGA	TTG	GAG	TCT	GCA	ACT	CGA	CTC	CAT	GAA
GTC	GGA	ATC	GCT	AGT	AAT	CGT	GGA	TCA	GAA	TGC	CAC	GGT	GAA	TAC
GTT	CCC	GGG	CCT	TGT	ACA	CAC	CGC	CCG	TCA	CAC	CAT	GGG	AGT	GGG
TTG	CAA	AAG	AAG	TAG	GTA	GCT	TAA	CCT	TCG	GGA	GGG	CGC	TTA	CCA
CTT	TGT	GAT	CAT	GAA	TGG	ATT	TG							

Figure 8.4 – The nucleotide sequence of the isolated and purified 16S gene. The 16S gene was sequenced by Eurofins MWG Operon (Ebersberg, Germany) using the 8f and 1510r primers. The forward and reverse sequencing results were pieced together to give the complete 16S sequence.

Accession no.	Description	% match
FM180568.1	<i>Escherichia coli</i> 0127:H6 E2348/69 complete genome, strain E2348/69	99%
AP012306.1	<i>Escherichia coli</i> str. K-12 substr. MDS42 DNA, complete genome	99%
CP002516.1	<i>Escherichia coli</i> KO11, complete genome	99%
AP012030.1	<i>Escherichia coli</i> DH1 (ME8569) DNA, complete genome	99%
CP002185.1	<i>Escherichia coli</i> W, complete genome	99%
FN649414.1	<i>Escherichia coli</i> ETEC H10407, complete genome	99%
CP001637.1	<i>Escherichia coli</i> DH1, complete genome	99%
CP001368.1	<i>Escherichia coli</i> O157:H7 str. TW14359, complete genome	99%
CP001396.1	<i>Escherichia coli</i> BW2952, complete genome	99%
CU928163.2	<i>Escherichia coli</i> UMN026 chromosome, complete genome	99%
AP009240.1	<i>Escherichia coli</i> SE11 DNA, complete genome	99%
CP001164.1	<i>Escherichia coli</i> O157:H7 str. EC4115, complete genome	99%
CP002797.2	<i>Escherichia coli</i> NA114, complete genome	99%
AM946981.2	<i>Escherichia coli</i> BL21(DE3), complete genome	99%
CP002167.1	<i>Escherichia coli</i> UM146, complete genome	99%
CP001509.3	<i>Escherichia coli</i> BL21(DE3), complete genome	99%
CP001969.1	<i>Escherichia coli</i> IHE3034, complete genome	99%
FN554766.1	<i>Escherichia coli</i> 042 complete genome	99%
CP001383.1	<i>Shigella flexneri</i> 2002017, complete genome	99%

Table 8.1 – A selection of the BLASTn results from the 16S gene search. The resultant 16S sequence was subjected to a BLASTn search of the ‘Nucleotide collection (nr/nt)’ database, with the ‘megablast’ option chosen. The results were sorted by ‘% match’ to give the best matches.

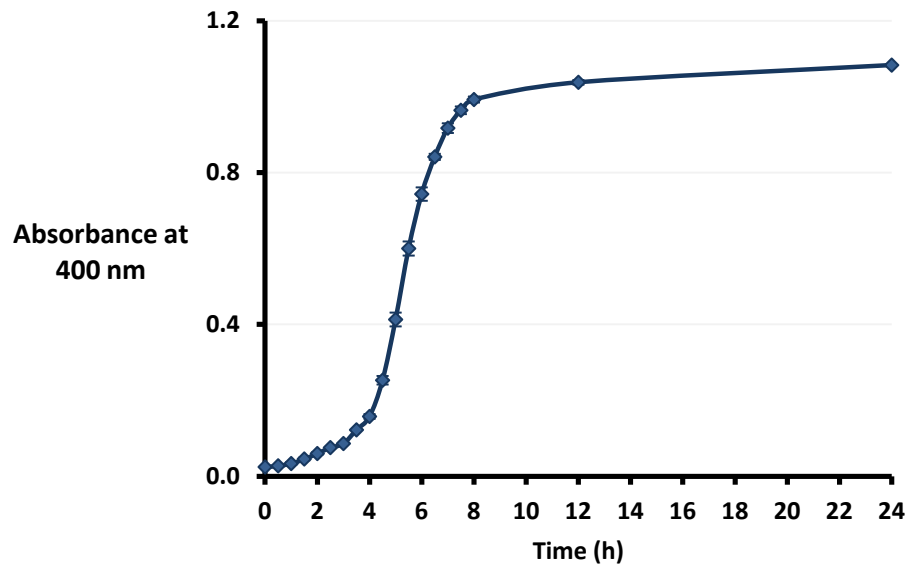


Figure 8.5 – *E. coli* C25 proliferates in cell culture media. *E. coli* C25 was grown overnight in TSB to obtain an active culture. A 1 in 500 dilution of the culture was prepared in basic cell culture media (DMEM + 4 mM glutamine) and the absorbance of the bacterial suspension was measured at 400 nm. The absorbance was subsequently taken every 30 min for the first 8 h and at the 12 and 24 h time points. Results are mean \pm SEM, n = 3.